44983

GENOTOXICITY ASSAYS FOR AMMONIUM PERCHLORATE

I. Salmonella/Microsome Mutagenesis

II. Mouse Lymphoma Cell Mutagenesis

III. *In Vivo* Mouse Bone Marrow Micronucleus Test

Final Report

January 20 through June 26, 1998

Study No. 6100-001

Submitted to

Mr. Michael Dourson
Study Sponsor
Perchlorate Study Group
TERA
4303 Hamilton Avenue
Cincinnati, OH 45223

GENOTOXICITY ASSAYS FOR AMMONIUM PERCHLORATE

I. Salmonella/Microsome Mutagenesis

II. Mouse Lymphoma Cell Mutagenesis

III. *In Vivo* Mouse Bone Marrow Micronucleus Test

Final Report

January 20 through June 26, 1998

Study No. 6100-001

Submitted to:

Mr. Michael Dourson
Study Sponsor
Perchlorate Study Group
TERA
4303 Hamilton Avenue
Cincinnati, OH 45223

From:

Sheela Sharma, M.S., Ph.D.
Study Director (Program Manager)

Pu Gao, M.D.
Principal Investigator

Cellular and Molecular Toxicology Program
Life Sciences and Toxicology Division
ManTech Environmental Technology, Inc.
A ManTech International Company

FOREWORD

This report is submitted in compliance with the requirements of the Contract and summarizes the work performed for the genotoxicology studies of ammonium perchlorate from January 20, 1998, through June 26, 1998, by the Cellular and Molecular Toxicology Program, ManTech Environmental Technology, Inc. (ManTech Environmental), for TERA, 4303 Hamilton Avenue, Cincinnati, OH 45223.

TABLE OF CONTENTS

FOREWORD

SUMMARY

Section I. INTRODUCTION

I.1 Salmonella/Microsome Mutagenesis Assay
I.2 Mouse Lymphoma Cell Mutagenesis Assay
I.3 *In Vivo* Mouse Bone Marrow Micronucleus Test

Section II. METHODS

II.1 Salmonella/Microsome Mutagenesis Assay
II.2 Mouse Lymphoma Mutagenesis Assay
II.3 *In Vivo* Mouse Bone Marrow Micronucleus Test

Section III. RESULTS

III.1 Salmonella/Microsome Mutagenesis Assay
III.2 Mouse Lymphoma Mutagenesis Assay
III.3 *In Vivo* Mouse Bone Marrow Micronucleus Test

Section IV. Good Laboratory Practice and Quality Assurance

Section V. References

APPENDICES

Appendix A. Salmonella/Microsome Mutagenesis Assay
Appendix B. Mouse Lymphoma Cell Mutagenesis Assay
Appendix C. *In Vivo* Mouse Bone Marrow Micronuclei Test
Appendix D. Protocol for the Genotoxicity Assays of Ammonium Perchlorate (AP)
Revised Protocol for the Genotoxicity Assays of Ammonium Perchlorate (AP)
Revised Protocol Page for Section VIII
Justification for Protocol Revisions

SUMMARY

Potential genotoxic effects of ammonium perchlorate (AP) in three short-term mutagenicity assays were investigated, which included the Salmonella/microsome mutagenesis assay (Ames test), the mouse lymphoma cell mutagenesis assay (L5178Y-TK test), and the *in vivo* mouse bone marrow micronucleus (MN test) induction assay.

In the Ames test, four strains (TA98, TA100, TA1535 and TA1537) were tested with and without the addition of an activation system (S9) at five concentrations (0.313, 0.625, 1.25, 2.5, and 5 mg/plate) of AP. The results of the assay indicate that AP did not cause any mutation in all four tester bacterial strains. In the mouse lymphoma cell mutagenesis assay, a single dose of 2.5 mg/mL showed some induction of mutation in the TK locus of a mouse lymphoma cell in the nonactivated group, but a repeat study indicated that this induction is not significant. In the *in vivo* mouse bone marrow micronucleus assay, there was no difference in MN induction in the AP-treated group at doses of 62.5, 125, 250, 500, and 1000 mg/kg in both male and female mice as compared to the negative control group. Therefore, based on the results obtained from the three studies, it is concluded that ammonium perchlorate is not a mutagenic agent in both bacterial and mammalian cells and causes no chromosomal damage in mouse bone marrow cells *in vivo*.

Section I

INTRODUCTION

Ammonium perchlorate (AP) is a rocket-fuel component that has been used in the U.S. Air Force and industry since the 1950s. AP is considered to be an explosive and is used as an oxidizer in solid fuel propellants. As AP has historically not been considered as a water contaminant, there is no state or federal standard to regulate its level in drinking water. Ammonium perchlorate contamination could be a serious problem, since it is believed to interfere with the thyroid gland's ability to use iodine for producing hormones. In a hormone deficient condition, normal metabolism, growth and development can be affected. Two large perchlorate-producing companies were located in Nevada, and ammonium perchlorate contamination in drinking water has become a serious environmental problem in the Lake Mead area in Nevada (McKinnon, 1998).

Data regarding AP toxicity are insufficient in the literature. In 1995, the U.S. Environmental Protection Agency reported a study of the effects of AP on bone marrow, but there are many questions about the chronic effects of perchlorate left unanswered by the existing data. On March 7, 1997, an International Toxicity Estimates for Risk (ITER) Peer Review Panel concluded that "the database for perchlorate was insufficient for development of a reference dose (RfD) and that additional studies should be conducted" (TERA, 1997).

The overall objective of the study is to determine the potential genotoxicity associated with the exposure to AP in three different *(in vitro* and *in vivo)* mutagenicity assays.

I.1 Samonella/Microsome Mutagenesis Assay (Ames Test)

The Salmonella/mammalian microsome revertant mutation system is a well-defined short-term assay for the detection of carcinogens/mutagens. It measures the reversion from a his^- (histidine dependent) to a his^+ (histidine independent) state induced by chemicals which cause base pair change or frameshift mutations in the genome of the organism. In this assay, bacteria are exposed to the test agent with and without a metabolic activation system (Aroclor-1254 induced rat liver S9 with co-factors) and plated onto minimum agar medium which is deficient in histidine. After incubation for 48 hours, revertant colonies are counted and compared with the number of spontaneous revertants in a untreated and/or vehicle control culture. The mutagenicity of the test agents is evident by the increase of revertants. The assay was performed in accordance with the EPA/TSCA Health Effect Testing Guideline 40 CFR 798-5265, 1995.

I.2 Mouse Lymphoma Cell Mutagenesis Assay (L5178Y-TK Test)

The L5178Y mouse-lymphoma assay is another short-term *in vitro* test system to detect quantitatively the mutagenic potential of compounds in mammalian cell cultures. Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principal deoxyribonucleotide monophosphates, only TMP does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under a normal growth condition, which serves as a regulator for DNA synthesis. If the TMP is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage"

enzyme, thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues.

This assay detects the forward mutations at the TK locus in a cell population which is heterozygous for the TK gene, using the L5178Y $TK^{+/-}$ cell line (Clive and Spector, 1975; Clive et al., 1983). The mutated cell line ($TK^{-/-}$) is resistant to trifluorothymidine (TFT), which is used as the selective agent. The basis for selection of the $TK^{-/-}$ mutants is the lack of any ability to utilize toxic analogs of thymidine, which enable only $TK^{-/-}$ mutants to grow in the presence of TFT. The assay is capable of identifying both structural changes (chromosomal mutations) and molecular changes (gene mutations) caused by a potential mutagen, and it can be used therefore to analyze the effect of these compounds in both somatic and germinal cells (Mitchell et al., 1988). The TFT resistant mutant colonies have a bimodal size distribution (Clive et al., 1979), which can be increased after chemical exposure. Large (λ) TFT resistant colonies have mutants affecting only the active TK gene, leaving the 11b chromosomes cytogenetically unaffected. Small (σ) TFT resistant colonies consist of cells having chromosomal aberration on chromosome 11b, which can be detected in conventionally stained and banded chromosomes (Blazak et al., 1989). This assay was carried out according to the EPA/TSCA Health Effect Testing Guideline 40 CFR 798-5300, 1995.

I.3 *In vivo* Mouse Bone Marrow Micronucleus Assay (MN Test)

The *in vivo* mammalian micronucleus test (Schmid, 1975), which detects the damage of chromosome or mitotic apparatus caused by chemicals, is used to examine the chromosome-damaging effect of the test agent. The damage of chromosome or mitotic apparatus caused by a clastogenic chemical in polychromatic erythrocyte (PCE) stem cells of treated animals is detected in this assay. Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during anaphase. After telophase, these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. Thus the micronucleus test can serve as a rapid screen for clastomatic agents and test articles that interfere with normal mitotic cell division, effecting spindle fiber function or formation. The assay is based on the increase in the frequency of micronucleated PCEs in bone marrow of the treated animals. The assay was conducted according to the EPA/TSCA Health Effect Testing Guideline 40 CFR 798-5395, 1995.

Section II

METHODS

The methodology for the three assays is described in the "Protocol for the Genotoxicity Assays of Ammonium Perchlorate" and included as Appendix D.

II.1 Salmonella/Microsome Mutagenesis Assay (Ames Test)

a. Materials

Salmonella typhimurium strains: Four tester strains (TA98, TA100, TA1535 and TA1537) were obtained from Dr. Bruce N. Ames, Dept. of Molecular and Cell Biology, University of California at Berkeley (Ames et al., 1975; Maron and Ames, 1983), and stored at -80°C to use in this assay.

Metabolic activation system: Aroclor 1254-induced rat (Sprague-Dawley adult male) liver *S9 homogenate* (Cat # 11-101, Lot # 0800) was purchased from Moltox (Boone, N.C.), and stored at -80°C. It was diluted with cofactors to make the standard S9 activation mixture. The S9 mixture contains 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, 4 mM NADP and 100 mM phosphate buffer (pH 7.4) and S9 (0.04 mL/mL mixture). The concentration (volume) of S9 is based on the historical data from the laboratory and the revised methods for the *Salmonella* mutagenicity test by Maron and Ames (1983). The S9 mixture was made fresh prior to use and kept on ice.

Growth medium: Bacto nutrient broth (Difco Laboratories, Cat # 0003-17-8, Lot # 116525JD), was prepared by dissolving 8 g powder and 5 g NaCl in 1 L of distilled water, which was sterilized and used for growing tester strains. The growth medium was routinely stored at 4°C.

Top agar: Top agar contains 0.6% Bacto agar (Difco Laboratory, Cat # 0140-05, Control # 715860) and 0.5 % NaCl in distilled water, which was autoclaved and stored at room temperature. Before plating, 10 mL of sterile 0.5 mM histidine/0.5 mM biotin solution was added to the melted top agar, kept at 45°C and used as an overlay on the minimal agar plate.

Minimal agar plate: The minimal agar was prepared by dissolving 1.5% Bacto agar (Difco Laboratory, Cat # 0140-05, Control # 715860) and 2% glucose in Vogel-Bonner medium E. Minimal agar plates were made by adding 30 mL of the minimal glucose agar medium onto a 100-mm x 15-mm bacterial plate. Vogel-Bonner medium E was prepared by dissolving 0.04 M $MgSO_4$, 0.52 M citric acid, 2.87 M K_2HPO_4, and 0.87 M $NaHNH_4$ in distilled water and sterilized. It was stored at 4°C.

Chemicals for genotypes confirmation:

Crystal violet (Fisher, Cat # C581, Lot # 870757): 0.1% dissolved in distilled water.
Histidine (Sigma, Cat # H-8125, Lot # 63H0202): 0.1 M dissolved in distilled water and sterilized.
Biotin (Sigma, Cat # B-4501, Lot # 34H0932): 0.5 M dissolved in distilled water and sterilized.
Ampicillin (Sigma Cat # A-9518 Lot # 85H0372): 8 mg/mL dissolved in 0.02N NaOH.

Positive control chemicals:

2-Aminofluorene (Sigma, Cat # A-9031, Lot # 12H2516): Dissolved in DMSO and further diluted with distilled water to 200 μg/mL, and 20 μg/plate was used.

Sodium azide (Sigma, Cat # S-2002, Lot # 113H0265): Dissolved in DMSO and further diluted with distilled water to 20 μg/mL, and 2 μg/plate was used.

9-Aminoacridine (Sigma Cat # A-1135, Lot # 56F0316): Dissolved in DMSO and further diluted with distilled water to 100 μg/mL and 10 μg/plate was used.

Test agent: Ammonium perchlorate: purchased from Aldrich (Cat # 20850-7 Lot # 03907LF) and stored at room temperature. It was dissolved in distilled water to make a stock solution of 50 mg/mL and further diluted to obtain desired concentrations.

b. Methods

Culturing of tester strains: The tester strains, frozen at -80°C were thawed, inoculated in nutrient broth and incubated in an environmental shaker incubator at 37°C for 12-15 hours to give the bacterial density of 1-2 x 10^9/mL. The bacteria were kept on ice before use.

Genotype confirmation: Genotypes of each strain were confirmed based on the methods described by Maron and Ames (1983) prior to the mutagenesis study, which included the requirement of histidine (his^-), the sensitivity to crystal violet (rfa mutation) and UV light (uvrB mutation), and the resistance to ampicillin (R factor). During the genotype confirmation assay, each tester strain was also plated onto the minimal agar plate and incubated for 48 hours, and the background lawn of growth was observed for evaluating the strain growth and for the occurrence of spontaneous revertants.

Range-finding assay: A preliminary range-finding assay was performed using strain TA100 to determine the optimal nontoxic test doses of AP. Ammonium perchlorate was freshly prepared in distilled water (0.005-50 mg/mL) and five log concentrations up to 5 mg/plate were tested with and without S9 activation. An aliquot (0.1 mL) of the culture was added to 2 mL of melted top agar, along with 0.1 mL of the test agent, and 0.5 mL of S9 mixture (in S9 plates only). The contents were then mixed and poured onto the surface of a minimum agar plate and spread out evenly. The plates were incubated at 37°C for 48 hours. The number of revertants per dish was counted manually or by an automatic Artek colony counter. Cultures were set up in triplicate; negative controls (spontaneous and solvent control [DMSO]) and a positive control (2-aminofluorene, 20 μg/plate) were also included.

Mutagenesis assay – Plate incorporation

All four tester strains (TA98, TA100, TA1535 and TA1537) were used in the mutagenesis assay. Ammonium perchlorate was freshly dissolved in sterile distilled water to obtain a dose range of 5, 2.5, 1.25, 0.625 and 0.315 mg/plate and tested in all four tester strains with and without S9 activation. The bacterium was cultured in nutrient broth at 37°C in an environmental shaker incubator for 12-15 hours. Next, 0.1 mL of the culture was added to 2 mL of melted top agar, along with 0.1 mL of the test agent and 0.5 mL of S9 mixture (in S9 plates only). The contents were then mixed and poured onto the surface of a minimum agar plate and spread out evenly. The plates were incubated at 37°C for 48 hours. The number of revertants per dish was counted manually or by an automatic colony counter. Cultures were set up in triplicate; negative controls (spontaneous and solvent (DMSO) control) and positive controls (2-

aminofluorene [with S9 for TA98 and TA100], sodium azide [without S9 for TA1535] and 9-aminoacridine [without S9 for TA1537]) were also included. A second independent experiment was also conducted.

II.2 Mouse Lymphoma Mutagenesis Assay

a. Materials

Cells: L5178Y $TK^{+/-}$ 3.7.2C cells, obtained originally from Dr. Donald Clive (former Burroughs Wellcome Co., Research Triangle Park, NC), were maintained in liquid nitrogen, or in suspension cultures at 37°C using Fischer's medium (F_{10p}) supplemented with 10% heat-inactivated horse serum and equilibrated with 5% CO_2 + 95% air. Cell counts were taken daily, and cultures were maintained at 3 x 10^5 cells/mL. To reduce the spontaneous frequency of $TK^{-/-}$ mutants, cells were treated with THMG (3 μg/mL thymidine, 5 μg/mL hypoxanthine, 0.1 μg/mL methotrexate, and 7.5 μg/mL glycine) for 24 hours and with THG (no methotrexate) for an additional 1-3 days, prior to AP exposure.

Medium: A 1.25 X stock solution of Fischer's medium was prepared by combining 1x powdered medium [10 L], sodium pyruvate (2.2 g), Pluronic F-68 (10 g), sodium bicarbonate (11.25 g), penicillin (10,000 units), and streptomycin (10 mg) in a final volume of 8 L (pH 6.8). Heat-inactivated horse serum (0, 100, or 200 mL) was added to 400 mL 1.25 X Fischer's medium, and the volume was adjusted to 500 mL, resulting in F_{0P}, F_{10p}, and F_{20P} medium, respectively. On the day of use, cloning medium (500 mL F_{20P}) was mixed with 50 mL of melted agar (3%) solution and kept at 47°C.

Test agent and controls: The test agent, AP and positive control agents, ethyl methanesulfonate (EMS) for the non-activated system and 3-methyl cholanthrene (3-MCA) for the activated system, and other chemicals were purchased from Sigma. On the day of use, AP was dissolved in medium and stock solutions (100 x) of controls were prepared in DMSO. Aroclor 1254-induced rat liver S9 homogenate was obtained from Moltox (Boone, N.C.), diluted with a cofactor mixture (250 mg of NADPH and 1500 mg of sodium isocitrate dissolved in 100 mL F_{0P}) prior to use, and kept on ice at -80°C.

b. Methods

Range finding: Preliminary range-finding experiments were performed in the presence and absence of S9 activation mixture, starting at 5 mg/mL AP as the top concentration followed by 10 lower doses over 5 orders of magnitude. The procedure used for range-finding experiments is identical to the one described for mutagenicity testing, with the exception that the cultures were terminated at 48 hours after exposure without cloning. Cell counts were determined 24 and 48 hours after treatment, and the suspension growth was determined from the cell counts as follows:

$$\textit{Suspension growth} = \frac{\textit{Day1 cell count}}{3x10^5} \; X \; \frac{\textit{Day2 cell count}}{3x10^5}$$

Mutagenicity assay: The mouse lymphoma (L5178Y $TK^{+/-}$) cells (6 x 10^6 cells in 10 mL medium for each culture) were treated with appropriate concentrations of AP in the presence (1 mL S9 + 3 mL cofactor mixture) and the absence of an S9 activation system. Positive controls (2.5 μg/mL 3-MCA for activated, and 250 nL/mL EMS for non-activated samples) and solvent (DMSO) control samples were also

included. Cells were equilibrated with 5% CO_2 : 95% air and incubated at 37°C for 4 hours in a roller drum at 20 rpm. After incubation, cultures were centrifuged (150 x *g* for 10 min), washed twice with fresh F_{10P} medium, resuspended in medium to obtain a final density of 3 x 10^5 cells/mL, and then incubated at 37°C in an atmosphere of 5% CO_2 : 95% air in the roller drum for 48 hours to enhance the expression of the newly induced mutations. On the cloning day (48 hours after exposure), the cells were diluted to 3 x 10^5 cells/mL and seeded onto soft agar medium in 100-mm dishes in triplicate. For each dose group, three cultures containing 200 cells/dish in non-selective medium (F_{10P}) were set up for viability measurement, and another set of three cultures with 1 x 10^6 cells/dish in selective medium containing TFT were used for mutant counting. Dishes were incubated at 37°C in an atmosphere of 5% CO_2, 95% air for 11 days before the counting and sizing of the mutants with an automatic colony counter. The mutant frequency was calculated and adjusted according the survival percentage.

II.3 *In vivo* Mouse Bone Marrow Micronucleus Assay (MN Test)

a. Materials

Animals: Swiss-CD-1 mice, male and female, 8-10 weeks old, were purchased from Charles River, Raleigh, NC. Upon receipt, animals were quarantined in the animal facility for one week as a quality control procedure. Animals were housed as five animals per cage for females, and one per cage for males. The experimental animals were identified by tail tattoos, each with a unique number. Food and water were provided ad libitum. Animals were randomized proportionately by body weight by using a computer software program two days before the AP treatment.

Chemicals: Cyclophosphamide (Sigma, Cat # C0768, Lot # 26H0473), dissolved in physiological saline (0.9% NaCl), was used as the positive control. The negative control was physiological saline (0.9% NaCl). The test agent, ammonium perchlorate (Aldrich, Cat # 20850-7 Lot # 03907LF), was dissolved in sterile distilled water before use.

b. Methods

Range finding: For the range-finding assay, 3 male and 3 female mice were used for each group. Animals were dosed with ammonium perchlorate (freshly prepared at concentrations ranging from 50-400 mg/mL) by gavage at 100 μL/10 g body weight to give an AP dose of 500, 1000, 2000 and 4000 mg/kg body weight. Negative control animals were given saline (100 μL/10 g body wt.) by gavage also. Animals were observed for signs of morbidity or mortality, and the observations were recorded at 10 min, 30 min and 24 hours after dosing. The survived animals were then dosed for three consecutive days. After 24 hours of the last dosing, mice were weighed and sacrificed, and bone marrow cells were collected. Animal mortality and the ratio of PCE and NCE in 1000 erythrocytes were used as indicators of AP toxicity. The highest nontoxic dose (1000 mg/kg body weight) was used as the top dose for the mutagenicity assay.

Slide preparation and microscopic observation: Mice were sacrificed by CO_2 inhalation, and bone marrow cells were collected by flushing the femur with a mixture of 1% sodium citrate and fetal bovine serum (70:30) and smears were made on slides and stained by May-Gruenwald/Giemsa solution. All slides were coded prior to scoring and scored blind. Next, 1000 red blood cells were randomly counted and the number of PCEs (polychromatic) and NCEs (normochromatic erythrocytes) were recorded separately.

appear as a densely stained small round body in the cytoplasm of the PCEs. The general size of the micronuclei is approximately 1/20-1/5 of the diameter of the PCEs. Totals for the number of PCEs, NCEs and miconucleated PCEs were recorded in a predesigned spreadsheet (Excel).

Mutagenicity Assay: For the mutagenicity assay, 5 male and 5 female mice in each group were dosed with AP or saline. AP was freshly dissolved in sterile distilled water to obtain concentrations ranging from 6.25 to 100 mg/mL and saline was used as the negative control. Both saline and AP were administered by gavage (100 μL /10 g body weight) for three consecutive days, with the AP dose ranging from 62.5 to 1000 mg/kg body weight. The positive control cyclophosphamide, dissolved in physiological saline, was given by i.p. injection as a single dose at a concentration of 2 mg/mL, in a volume of 100 μL/10 g body weight to obtain a dose of 20 mg/kg/ body weight. After 24 hours of the last dosing, mice were sacrificed, and bone marrow cells were collected and processed as described before. The frequency of micronucleated cells were observed by counting 1000 PCEs per animal. The ratio of PCE and NCE in 1000 erythrocytes was used as the indicator of toxicity.

Section III

RESULTS

III.1 Salmonella/Microsome Mutagenesis Assay (Ames Test)

The raw data for the Ames Test are attached as Appendix A and the salient results are summarized as follows.

a. Genotype Identification

Different genotypes of the tester strains were verified by the standard procedure of Ames et al. (1975) prior to the study. Results (see Table III-1) indicated that all the tester strains were qualified for the study.

Table III-1. Genotype Confirmation of Tester Strains

Genotypes	TA98	TA100	TA1535	TA1537
Histidine requirement	+	+	+	+
rfa mutation	+	+	+	+
uvrB mutation	+	+	+	+
R factor	+	+	—	—
Spontaneous revertants	38±2	156±3	17±1	10±1

b. Dose Selection for AP

Five log doses (0.0005-5 mg/plate) of AP were tested in TA100 for the dose selection and the results are listed in Table III-2. No toxicity was observed in any of the doses tested. Therefore, in the mutagenesis assay, 5 mg/plate was selected as the top dose followed by four twofold dilutions.

Table III-2. Results of AP Dose Selection Assays

Treatment	Revertants/plate (Mean±SD) S9+	Revertants/plate (Mean±SD) S9-
DMSO	99.3±3.39	103±10.03
2-AF (20 μg)	1076.6±39.85	
AP (mg/plate)		
5	139.33±17.01	130.66±20.4
0.5	142.33±5.31	116±16.3
0.05	131.6±29.63	140±12.08
0.005	121±17.37	128.33±8.57
0.0005	121.6±1.88	128.33±19.13
Spontaneous	131.3± 0.9	123 ± 1.03

c. Mutagenicity Assay

Results: The results of mutagenicity assays in two independent experiments with four tester strains (TA98, TA100, TA1535, and TA1537) are summarized in Table III-3. The data are expressed as the average revertant number per plate from the triplicates. The results indicate that ammonium perchlorate did not increase the revertant numbers in all four tester strains at all the five concentrations tested in comparison to the solvent (spontaneous) control.

Discussion: The data generated in this assay is acceptable because (1) the spontaneous revertant number is within the normal range (TA98: 30-50, TA100: 120-200, TA1535: 15-25 and TA1537: 5-10) reported in the literature and the laboratory's data range (TA98: 27.5 ± 4.9 - 35.0 ± 6.8, TA100: 140.8 ± 18.6 - 146.16 ± 21.0, TA1535: 11.8 ± 4.4 - 15.7 ± 3.4, TA1537: 8.8 ± 2.0 - 8.2 ± 1.2), (2) five nontoxic 1:1 serial doses were used, (3) the positive mutagens such as sodium azide for TA1535 and 9-aminoacridine for TA1537 in non-activating systems yielded a significant increase (64- to 69-fold increase) in the number of revertants as compared to solvent control. Similarly, 2-aminofluorene, the positive control for both TA98 and TA100, in the activated system also yielded a significant increase (46- to 47-fold) in the number of revertants. In conclusion, the positive controls adequately demonstrated the sensitivity of the assay because (a) the positive response with 2-AF showed that the S9 system was capable of activating mutagens and (b) the sensitivity of each strain to detect mutagens was demonstrated.

d. Conclusion

The above results indicate that ammonium perchlorate is not mutagenic in the Salmonella/microsome mutagenesis assay.

Table III-3 Mutagenesis Assay of Ammonium Perchlorate in Ames Test

Treatment (Dose/Plate)	Revertants/Plate (mean ±SD) TA98 S9+	TA98 S9-	TA100 S9+	TA100 S9-	TA1535 S9+	TA1535 S9-	TA1537 S9+	TA1537 S9-
Experiment # 1								
DMSO	24.67±1.53	21.67±4.04	147.67±18.82	140.66±11.01	13.33±3.51	14.00±1.73	6.00±1.00	7.33±1.53
2-AF (20 µg)	1140.67±28.59		1224.33±45.65					
Sod. azide (2 µg)						960.00±31.19		
9-Am. acridine (10 µg)								167.33±29.48
Ammonium perchlorate (mg)								
5	31.00±6.00	27.00±4.00	181.67±3.51	155.33±20.26	15.33±1.53	13.67±4.16	7.00±1.73	8.67±1.53
2.5	25.00±1.00	28.00±1.73	171.33±16.04	178.33±6.66	15.67±3.79	12.33±2.88	11.00±2.00	8.00±2.00
1.25	25.00±2.65	33.67+5.03	179.00±20.66	162.67±25.33	15.67±0.58	12.33±3.22	9.67±3.06	8.33±0.58
0.625	36.67±3.22	23.00±3.61	160.33±18.48	179.00±15.62	14.00±2.00	19.00±1.00	9.67±3.06	10.33±1.16
0.3125	25.00±2.65	24.00±1.73	176.33±22.75	155.00±22.52	16.67±2.08	18.33±2.08	8.00±2.00	10.33±2.31
Spontaneous	38.7±3.05	31.66±0.57	161.00±20.00	157.66±4.62	13.66±5.50	16.33±2.08	7.66±4.01	9.60±1.23
Experiment #2								
DMSO	32.00±5.29	27.00±2.64	123.67±8.02	124.00±5.29	19.67±4.16	14.00±1.00	9.00±1.00	7.33±0.58
2-AF (20 µg)	1516.67±82.4		1394.00±36.17					
Sod. azide (2 µg)						895.33±71.12		
9-Am. acridine (10 µg)								95.67±3.22
AP (mg)								
5	40.00±2.65	28.33±1.53	198.00±2.65	140.33±38.68	14.67±4.04	12.67±3.22	5.67±1.16	8.33±1.53
2.5	34.33±3.51	26.67±3.22	122.33±11.06	113.33±7.02	10.33±0.58	15.00±2.65	6.67±2.08	6.67±1.53
1.25	40.67±5.77	23.67±1.16	111.00±1.00	109.00±2.65	17.67±3.06	15.67±1.53	6.33±1.53	6.67±0.58
0.625	42.67±4.16	28.67±2.31	124.67±5.51	113.33±13.01	11.00±1.00	14.00±1.00	8.67±0.58	6.67±0.58
0.3125	35.33±3.51	28.33±0.58	113.33±5.51	122.33±4.04	11.33±0.58	14.67±1.53	6.00±1.00	7.00±2.00
Spontaneous	31.00±7.93	23.33±0.58	131.33±4.50	124.00±5.29	10.00±2.00	14.33±4.04	6.66±1.15	8.00±4.58

III.2 Mouse Lymphoma Mutagenesis Assay

The raw data for the L5178Y-TK test are attached as Appendix B, and the results are summarized as follows.

a. Range Finding

Results of the preliminary dose selection experiment are given in Table III-4. The toxicity was remarkably high in the activated system, 99.5% at the highest concentration (5 mg/mL) compared to 66.6% toxicity at the same concentration in the non-activated system. Based on the results, the top concentrations for the mutagenesis assay were 5 and 2.5 mg/mL for the non-activated and the activated system, respectively, followed by four serial, fivefold dilutions.

Table III-4. Range Finding Assay With Ammonium Perchlorate

AP concentration (µg/mL)	NONACTIVATED (-S9) SYSTEM				ACTIVATED (+S9) SYSTEM			
	Daily growth[a] Day 1	Daily growth[a] Day 2	Cumulative cell counts[b] (10^6/mL)	Relative suspension growth[c]	Daily growth[a] Day 1	Daily growth[a] Day 2	Cumulative cell counts[b] (10^6/mL)	Relative suspension growth[c]
Medium	6.37	5.67	10.82	100.00	4.07	5.25	6.41	100.00
0.25	5.37	5.38	8.67	80.08	3.53	5.70	6.04	94.33
0.5	5.92	4.97	8.82	81.45	5.37	4.92	7.92	123.59
2.5	3.73	5.47	6.12	56.57	4.67	5.93	8.31	129.69
5	4.33	4.90	6.37	58.85	1.43	6.58	2.83	44.20
25	2.83	4.33	3.68	34.03	3.72	4.67	5.20	81.24
50	3.52	5.43	5.73	52.96	3.27	4.90	4.80	74.97
250	2.53	5.12	3.89	35.93	3.73	5.43	6.09	95.01
500	2.83	4.77	4.05	37.43	3.00	4.47	4.02	62.76
2500	2.38	4.77	3.41	31.49	1.42	2.13	0.91	14.16
5000	2.60	4.63	3.61	33.39	0.13	0.77	0.03	0.48
Positive Control	4.00	4.57	5.48	50.63	3.23	5.67	5.50	85.82

a: Daily growth= Observed cell conc./initial seeding conc. (3 x 10^5 cells/mL)
b: Cumulative cell counts (CCC) = Initial seeding conc. x Day 1 growth x Day 2 growth
c: Relative suspension growth= CCC in treated group/CCC in medium control

b. Mutagenesis Assay

Results: The summary of results of AP in both activated and non-activated systems are presented in Table III-5A. The difference in the mutation frequency between treated and control samples was evaluated by the two-tail Student's *t*-test. In the non-activated samples, an increase in mutation frequency was observed at a single concentration (2.5 mg/mL), which appeared to be statistically significant ($p < 0.05$). Therefore, a repeat assay of AP in the non-activated system was conducted in order to examine the reproducibility of the data from a single dose and the results are shown in Table III-5B. As is evident from the results, there was no increase in mutation frequency at 2.5 mg/mL compared to the medium control. On the contrary, the positive control, EMS, showed a four to sevenfold statistically significant increase in mutation frequency as compared to the negative (DMSO) solvent control ($p < 0.05$).

In the activated system (+S9), there was no increase in mutation frequency, whereas the positive control for the activated system, 3-MCA, showed a twofold statistically significant increase in mutation frequency ($p < 0.05$).

Table III-5A. Mouse Lymphoma Mutagenesis Assay With Ammonium Perchlorate

Treatment	Concentration (mg/mL)	Cloning Data VC/dish[a]	Cloning Data TFT/dish[a]	CFE[b]	RCFE[c]	Mf[d] ($x10^6$)	RMF[e] (%)
NON-ACTIVATED (-S9) SYSTEM							
Medium	-	180.0 ± 14.4	89.2 ± 8.0	0.90	1.00	99.1 ± 8.9	100.0
AP	0.025	187.6 ± 2.5	81.7 ± 4.3	0.94	1.04	87.1 ± 4.6	87.8
AP	0.05	134.3 ± 13.9	56.0 ± 7.5	0.67	0.75	83.4 ± 11.2	84.1
AP	0.25	179.7 ± 9.0	72.8 ± 15.7	0.90	1.00	81.0 ± 17.5	81.7
AP	0.5	189.7 ± 21.7	103.2 ± 14.7	0.95	1.05	108.8 ± 15.5	109.8
AP	2.5	128.7 ± 1.2	81.9 ± 3.4	0.64	0.71	127.3 ± 5.2	128.4*
AP	5.0	164.6 ± 15.7	86.7 ± 13.0	0.82	0.91	105.3 ± 15.8	106.3
DMSO		182.2 ± 25.0	70.0 ± 0.5	0.91	1.00	51.2 ± 44.4	100.0
EMS	250 nL/mL	197.3 ± 4.7	303.0 ± 13.5	0.99	1.08	307.1 ± 13.7	399.7*
ACTIVATED SYSTEM (+S9)							
Medium	-	183.7 ± 6.7	94.2 ± 13.2	0.92	1.00	102.6 ± 14.4	100.0
AP	0.025	165.1 ± 2.9	56.3 ± 2.3	0.83	0.90	68.2 ± 2.8	66.5
AP	0.05	252.8 ± 0.5	104.0 ± 11.2	1.26	1.38	82.3 ± 8.8	80.2
A P	0.25	247.6 ± 14.9	108.3 ± 10.3	1.24	1.35	87.5 ± 8.3	85.3
AP	0.5	207.6 ± 20.0	67.9 ± 8.8	1.04	1.13	65.4 ± 8.4	63.8
AP	2.5	286.8 ± 22.2	82.1 ± 3.1	1.43	1.56	57.03 ± 2.1	55.8
DMSO		193.8 ± 3.0	89.0 ± 2.6	0.97	1.00	91.9 ± 2.7	100.0
3-MCA	2.5 μg/mL	193.8 ± 10.4	176.9± 6.2	0.97	1.00	182.6 ± 6.4	198.8*

a: averages of three dishes
b: colonies on 'VC' dish /200 cells
c: CFE of treated/CFE of untreated
d: (colonies of 'TFT' dish/CFE)/10^6 cells
e: (MF of treated/MF of nontreated) X 100
* significant ($p < 0.05$)

TABLE III-5B. Mouse Lymphoma Mutagenesis Assay with AP (-S9) - Repeat Assay

Treatment	Concentration (mg/mL)	Cloning Data VC/dish[a]	TFT/dish[a]	CFE[b]	RCFE[c]	Mf[d] ($x10^{-6}$)	RMF[e] (%)
NON-ACTIVATED (-S9) SYSTEM							
Medium	-	150.6±3.4	72.7±7.5	0.75	100	96.48±9.96	100
AP	0.025	211.0±31.5	85.7±9.3	1.06	140.1	81.2±8.8	84
AP	0.05	214.8±5.9	68.7±8.1	1.07	142.6	63.95±7.58	66
AP	0.25	191.7±5.9	74.0±6.6	0.96	127.2	77.22±6.8	80
AP	0.5	137.0±4.0	74.3±6.4	0.69	90.9	108.52±9.38	102
AP	2.5	190.0±10.4	85.7±2.1	0.95	126.1	90.18±2.19	93
AP	5.0	173.3±5.1	87.0±3.0	0.87	115.1	100.38±3.46	104
DMSO		176.3±6.8	72.3±7.1	0.88	100	82.04±8.04	85
EMS	250 nl/ml	111.3±14.0	388.3±31.6	0.56	63.1	697.3±56.7	723

a: averages of three dishes
b: colonies on 'VC' dish /200 cells
c: CFE of treated/CFE of untreated
d: (colonies of 'TFT'dish/CFE)/10^6 cells
e: (MF of treated/MF of nontreated)*100
* significant ($p < 0.05$)

Size distribution

The distributions of the mutant size for both positive controls (EMS and 3-MCA) are presented in Figure III-1 and III-2. The mutants induced by 2.5 nL/mL EMS (non-activated system) showed a single peak of mutants at sizes of 0.5 - 0.7 mm. The other positive control, 3-MCA (2.5 μg/mL, activated system), had two peaks, one indicating mutant size of 0.1 mm and the other with sizes ranging from 0.6 to 0.7 mm.

Discussion: The data generated in the two systems are considered acceptable because of the following.

(1) The spontaneous mutation frequency is in the normal range as reported in the literature and also within the laboratory's historical range. For example, the normal range of background frequencies for assays performed with different cell stocks is cited in literature as 20 - 120 x 10^{-6} (Brusick, 1994). Our background frequency during ML assay validation ranged from 71.3 (+S9) to 89.5 (-S9). Historically, the frequency values in untreated cultures ranged from 66 to 90 ± 8 (-S9) and from 70 to 92 ± 9.4 (+S9). Even though our values in the current studies are 99.1 ± 8.9 and 96.5 ± 9.96 for nonactivated and 102.6 ± 14.4 for activated, they are still in the acceptable range as cited by Brusick (1994). It is also noteworthy to mention that the background frequencies for solvent control (DMSO) in the two experiments (-S9) are quite low (51.2 - 82.04) (see Tables III-5A and III-5B).

(2) The test system is sensitive to the two known mutagens, EMS and 3-MCA, as indicated by the results. The normal range of increase in mutation frequency for methylmethane sulfonate (-S9) at -10.4 μL/mL is from 2- to 8-fold (Brusick, 1994), whereas our results with ethylmethane sulfonate (a similar

chemical mutagen) is from 4- to 7-fold at a much lower dose (0.25 μL/mL or $\sim 1/40^{th}$ of MMS dose). Our ML assay validation data (not included) and historical data show, respectively, a 6.7-fold and a 4- to 5.7-fold increase in mutation frequencies compared to the untreated control. 3-Methyl cholanthrene, the positive control for the activated system (+S9), shows a 2-to 10-fold increase in mutation frequency at 4.0 μg/mL (Brusick, 1994), whereas our results at a lower concentration (2.5 μg/mL) still show a 2-fold increase compared to the DMSO control. Again, our ML validation data indicates a 3.2-fold increase at the same dose, and the historical data indicates 2-to 3.4-fold increase.

(3) For acceptable experiments, the cloning efficiencies of the solvent control cultures should be greater than 70% (Clive et al., 1979; Turner et al., 1984). Our experiments indicate that the average cloning efficiencies in medium control and DMSO control cultures were 86% and 94%, respectively.

b. Conclusions

Based on the results from the two systems (–S9 and +S9), it is concluded that AP is not mutagenic in the mouse lymphoma cell assay, as there was no statistically significant induction in the number of mutant colonies after AP treatment. On the other hand, both the positive controls, EMS and 3-MCA, exhibited statistically significant induction of mutant colonies with characteristic large mutant sizes ranging from 0.5 to 0.7 mm. 3-MCA also had a single peak of small mutants (0.1 mm). It has been suggested that small colony mutants (σ) may arise from the induction of chromosomal damage, while the large colony mutants (λ) arise from gene mutation (Clive et al., 1979; Hozier et al., 1982; Blazak et al., 1989; Moore et al., 1985).

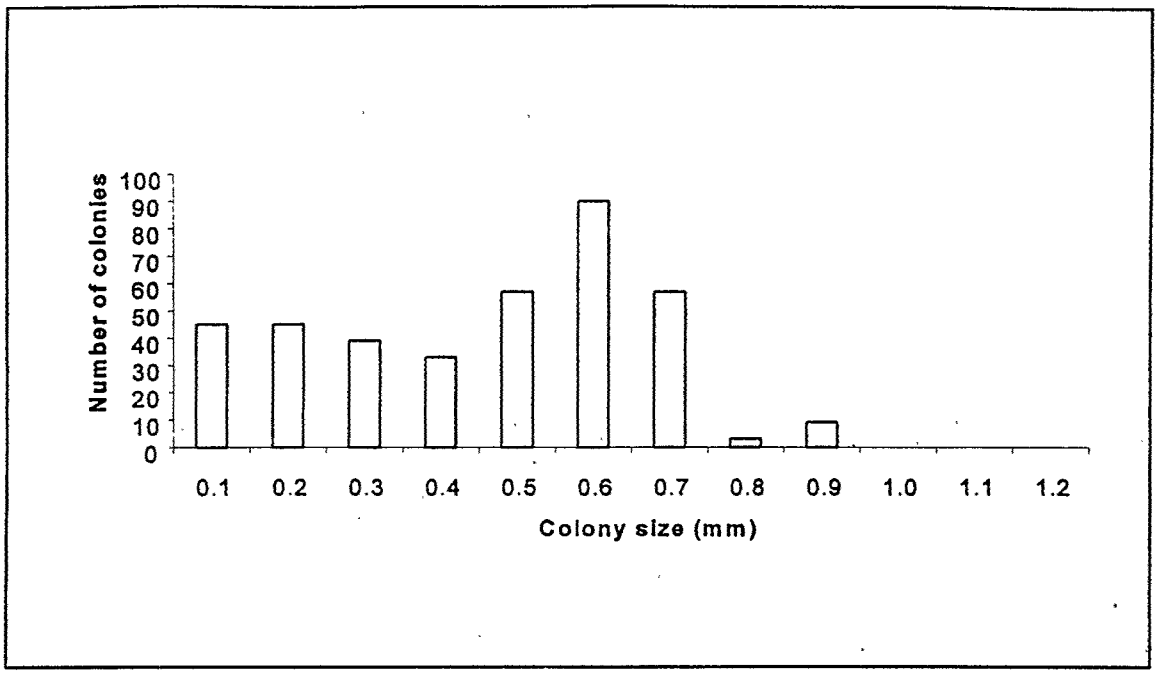

Figure III-1. Size distribution of L5178Y $TK^{-/-}$ mutant colonies after EMS (250 nL/mL) treatment (non-activated system)

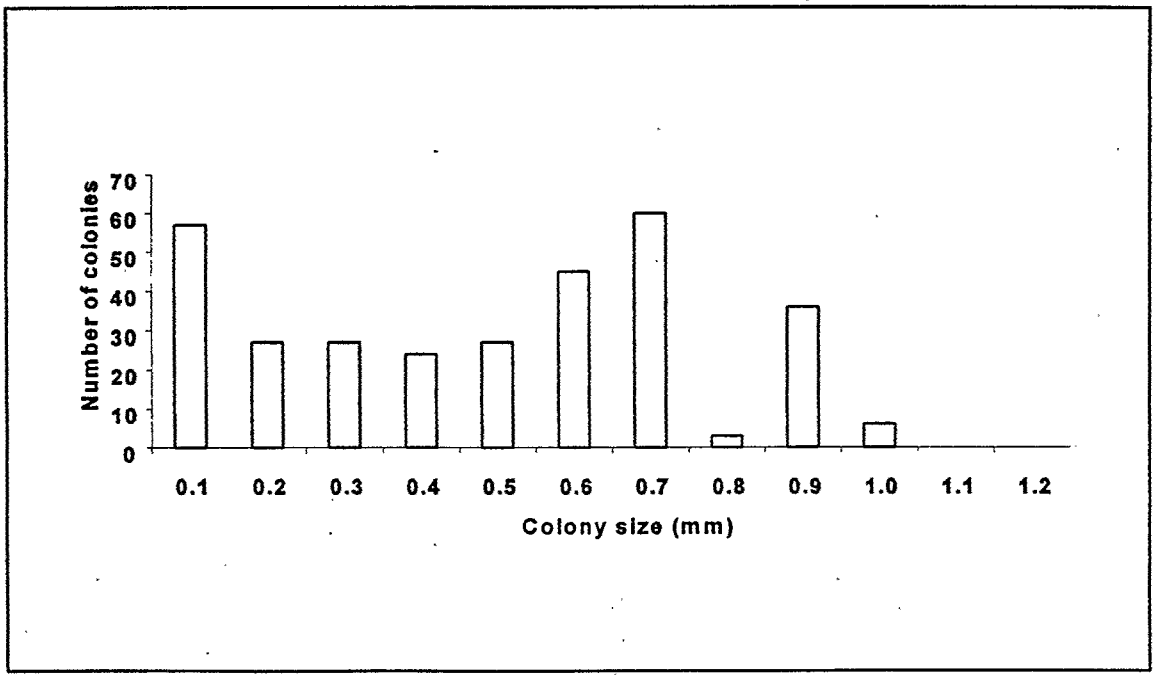

Figure III-2. Size distribution of L5178Y $TK^{-/-}$ mutant colonies after 3-MCA (2.5 μg/mL) treatment (activated system)

III.3 *In vivo* Mouse Bone Marrow Micronucleus Assay (MN Test)

The raw data for the MN test are attached as Appendix C and the results are summarized as follows.

a. Range Finding

A range-finding assay was conducted with AP at doses of 500-4000 mg/kg body weight. Thirty minutes after ammonium perchlorate was administered by gavage, 3 male and 2 female mice died in the 4000-mg/kg group. After 24 hours, two male and two female mice died in the 2000-mg/kg group. The body weights of the remaining mice were taken, and there was no difference between the treated and the control group (male: 30.4 - 32.1 $\pm$ 0.87 g in the treated group, 31.2 - 32.0 $\pm$0.4 g in the control group; female: 24.6 - 25.3 $\pm$ 0.21 g in the treated group, 24.2 - 25.2 $\pm$ 0.5 g in the control group). They were dosed for another 2 consecutive days with AP at concentrations of 1000 and 500 mg/kg. All mice survived the treatment, and 24 hours after the last dosing, they were sacrificed, bone marrow cells were collected, and PCEs and NCEs were counted. There was no significant difference in the ratio of PCEs and NCEs (determined in 1000 cells) in the treatment group compared to the control group. Therefore, 1000 mg/kg was selected as the top dose, followed by four 2-fold dilutions in the mutagenesis assay.

b. Mutagenicity Assay

Results: Based on the substantial toxicity observed at only twice the highest tested dose (2000 mg/kg), a sufficiently high dose (1000 mg/kg) was tested in the mutagenicity study, even though no cytotoxicity was observed in the target cells (as evidenced by the ratio of PCEs and NCEs). The results of the effect of ammonium perchlorate on the bone marrow cell micronucleus induction in male and female mice are presented in Table III-6 and III-7, respectively. The background frequencies of micronucleated PCE are usually in the range of 0.1-0.3%. The background of micronucleated PCE frequency in our study was 0.14% in males and 0.19% in females. The positive control, CP, increased the frequency of micronucleated PCE by about 10.4-fold in males ($p < 0.0001$) and 8.3-fold in female mice ($p < 0.0001$). On the other hand, ammonium perchlorate did not induce any micronuclei in mouse bone marrow cells in any of the treatment groups. There was no difference between the treatment group and the negative control group (ANOVA, Student *t*-test, $P > 0.05$).

Discussion: The data generated is considered valid because the background frequency of micronucleated cells is in the normal range from the literature (0.1 - 0.3%) and the historical data from the laboratory (males, 0.14 to 0.28 $\pm$ 0.1; females, 0.19 to 0.3 $\pm$ 0.1). Furthermore, the test system is highly sensitive to the positive mutagen cyclophosphamide because it caused a 10.4-fold and a 8.3-fold increase in the micronucleated frequency in males ($p < 0.0001$) and females ($p < 0.0001$), respectively.

c. Conclusion

The above results indicate that, under the experimental conditions, ammonium perchlorate in any of the concentrations did not show any statistically significant dose-related increase in the number of micronucleated PCEs or a significant increase in the micronucleated cell frequency in the Swiss CD-1 mice polychromatic erythrocyte system. Therefore, AP is not mutagenic in the *in vivo* mouse bone marrow cell micronucleus assay.

Table III-6. Effect of Ammonium Perchlorate on Micronucleus Induction in Male Mouse Bone Marrow Cells

Treatment mg/kg	No. Of mice per dose	% MNPCE ± SD	PCE/NCE ± SD
0 (physiological saline)	5	0.14 ± 0.06	0.61 ± 0.09
AP, 62.5	5	0.17 ± 0.07	0.62 ± 0.13
AP, 125	5	0.13 ± 0.05	0.59 ± 0.06
AP, 250	5	0.09 ± 0.003	0.66 ± 0.05
AP, 500	5	0.1 ± 0.003	0.70 ± 0.04
AP, 1000	5	0.12 ± 0.06	0.68 ± 0.04
CP, 20	5	1.45 ± 0.11	0.62 ± 0.08

Table III-7. Effect of Ammonium Perchlorate on Micronucleus Induction in Female Mouse Bone Marrow Cells

Treatment mg/kg	No. of mice per dose	% MNPCE ± SD	PCE/NCE ± SD
0 (physiological saline)	5	0.192±0.06	0.54±0.11
AP, 62.5	5	0.13±0.05	0.54±0.05
AP, 125	5	0.18±0.045	0.68±0.03
AP, 250	5	0.12±0.04	0.63±0.13
AP, 500	5	0.16±0.07	0.68±0.14
AP, 1000	5	0.17±0.08	0.69±0.08
CP, 20	5	1.59±0.07	0.59±0.06

Section IV

GOOD LABORATORY PRACTICES AND QUALITY ASSURANCE

All assays were conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA / TSCA) Good Laboratory Practice (GLP) standards as defined in the Federal Register (40 CFR Part 792, 1992) and the TSCA Test Guidelines (40 CFR, 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1995). All the procedures were performed in accordance with the Standart Operating Procedure (SOPs) of ManTech Environmental for the Salmonella/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis and *in vivo* mouse bone marrow cell micronucleus test.

The quality assurance auditors Ms. Susane Nemecz, Ms. Betty Wilkinson and Mr. James Macri documented inspections on procedures used in this study.

	Date of inspection	Items /activity inspected
A.	Salmonella/microsome mutagenesis assay:	
	January 27, 1998	Mutagenesis assay (Plate incorporation)
	Susane Kdo Quality Assurance Auditor	5|12|98 Date
B.	Mouse lymphoma cells mutagenesis assay:	
	February 17, 1998 May 5, 1998	Range finding assay Mutagenesis assay
	Quality Assurance Auditor	5·12·98 Date
C.	*In vivo* mouse bone marrow micronucleus test:	
	March 2, 1998 March 5, 1998 April 9, 1998	Chemical exposure Collection of bone marrow cells, make smears Scoring of micronuclei and PCE/NCE
	BP Wilkinson Quality Assurance Auditor	5·12·98 Date

Section V

REFERENCES

Ames BN., McCann J., Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutat Res* 31:47-364.

Blazak WF., Los FJ., Rudd CJ., Caspary WJ. 1989. Chromosome analysis of small and large L5178Y mouse lymphoma cell colonies: comparison of trifluorothymidine-resistant and unselected cell colonies from mutagen-treated and control cultures. *Mutat Res* 224:197-208.

Brusick D. 1994. Genetic Toxicology. In: *Principles and Methods of Toxicology, Third Edition*, edited by A. Wallace Hayes, pp. 545-577. Raven Press, Ltd., New York.

Clive D. and Spector JFS. 1975. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutat Res* 37:17-29.

Clive D., Johnson KO., Spector JFS., Batson AG., Brown MMM. 1979. Validation and characterization of the L5178Y/TK$^{+/-}$ mouse lymphoma mutagenesis assay system. *Mutat Res* 59:61-108.

Clive D., McCuen, R., Spector JFS., Piper C., Mavournin KH. 1983. Specific gene mutations in L5178Y cells in culture. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 115:225-251.

EPA. 1992. EPA/TSCA Good Laboratory Practice. 40 CFR Part 792.

EPA. 1995. EPA/TSCA Test Guidelines. 40 CFR 798-5265.

EPA. 1995. EPA/TSCA Test Guidelines. 40 CFR 798-5300.

EPA. 1995. EPA/TSCA Health Effect Testing Guideline. 40 CFR 798-5395.

Hozier J., Sawyer J., Clive D., Moore M. 1982. Cytogenetic distinction between the *tk*$^{+}$ and *tk*$^{-}$ chromosomes in the L5178Y *tk*$^{+/-}$ cell line. **Mutat Res** 105:451-456.

Maron DM. and Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. *Mutat Res* 113: 173-215.

McKinnon S. 1998. Perchlorate levels surge in Lake Mead. *Las Vegas Review Journal*, January 09.

Mitchell AD., Myhr BC., Rudd CJ., Caspary WJ., Dunkel VC. 1988. Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Methods used and chemicals evaluated. *Environ Molec Mutagen* 12 Suppl. 13:1-18.

Schmid W. 1975. The micronucleus test. *Mutat Res* 31: 9-15.

TERA. 1997. Results of the Perchlorate Study Protocol Review Meeting. *TERA News*: Perchlorate Study Protocol Peer Review May 20, 1997 Summary Meeting Notes.

Turner NT., Batson AG., Clive D. 1984. Procedures for the L5178Y/TK$^{+/-}$→ TK$^{-/-}$ mouse lymphoma cell mutagenicity assay. In Kilby B, Legatro M, Nichols W, Ramel C (eds): "Handbook of mutagenicity test procedures, Ed. 2" Amsterdam: Elsevier/North Holland, pp 239-268.

APPENDIX A

RAW DATA OF SALMONELLA/MICROSOME MUTAGENESIS ASSAY

Appendix - A1
Ames Test - Strain TA100
Range-finding

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		98	104	96	99.33	3.39
2-AF	20 ug	1122	1025	1083	1076.66	39.85
AP	mg					
	5	135	121	162	1369.33	17.01
	0.5	136	149	142	142.33	5.31
	0.05	105	117	173	131.66	29.63
	0.005	140	98	125	121.00	17.37
	0.0005	123	119	123	121.66	1.88
Spontaneous	0.00	132	133	131	131.31	0.90
S9-						
DMSO		94	98	117	103.00	10.03
AP	mg					
	5	109	125	158	130.66	20.40
	0.5	93	129	126	116.00	16.30
	0.05	147	123	150	140.00	12.08
	0.005	128	139	118	128.33	8.57
	0.0005	111	119	155	128.33	19.13
Spontaneous	0.00	124	125	123	123.31	1.03

Appendix - A1
Ames Test - Strain TA98
Experiment # 1

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		26	23	25	24.67	1.53
2-AF	20 ug	1134	1172	1116	1140.67	28.59
AP	mg					
	5	25	31	37	31.00	6.00
	2.5	24	25	26	25.00	1.00
	1.25	23	24	28	25.00	2.65
	0.625	38	39	33	36.67	3.22
	0.313	24	23	28	25.00	2.65
Spontaneous	0.0	38	42	38	38.7	3.05
S9-						
DMSO		21	18	26	21.67	4.04
AP	mg					
	5	27	31	23	27.00	4.00
	2.5	29	29	26	28.00	1.73
	1.25	39	33	29	33.67	5.03
	0.625	20	22	27	23.00	3.61
	0.313	23	26	23	24.00	1.73
Spontaneous	0.00	31	32	32	31.66	0.57

Appendix - A1
Ames Test - Strain TA98
Experiment # 2

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		26	36	34	32.00	5.29
2-AF	20 ug	1537	1426	1587	1516.67	82.40
AP	mg					
	5	39	38	43	40.00	2.65
	2.5	34	38	31	34.33	3.51
	1.25	34	44	44	40.67	5.77
	0.625	38	44	46	42.67	4.16
	0.313	35	32	39	35.33	3.51
Spontaneous	0.00	37	34	22	31.00	7.93
S9-						
DMSO		25	26	30	27.000	2.646
AP	mg					
	5	30	27	28	28.33	1.53
	2.5	29	28	23	26.67	3.22
	1.25	23	23	25	23.67	1.16
	0.625	30	30	26	28.67	2.31
	0.313	28	29	28	28.33	0.58
Spontaneous	0.00	23	23	24	23.33	0.58

Appendix - A2
Ames Test - Strain TA100
Experiment # 1

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		126	157	160	147.67	18.82
2-AF	20 ug	1172	1245	1256	1224.33	45.65
AP	mg					
	5	178	185	182	181.67	3.51
	2.5	170	156	188	171.33	16.04
	1.25	198	157	182	179.00	20.66
	0.625	171	139	171	160.33	18.48
	0.313	151	195	183	176.33	22.75
Spontaneous	0.00	161	181	141	161.00	20.00
S9-						
DMSO		148	128	146	140.667	11.015
AP	mg					
	5	178	149	139	155.33	20.26
	2.5	175	174	186	178.33	6.66
	1.25	182	172	134	162.67	25.33
	0.625	197	169	171	179.00	15.62
	0.313	154	178	133	155.00	22.52
Spontaneous	0.00	163	155	155	157.66	4.62

Appendix - A2
Ames Test - Strain TA100
Experiment # 2

S9+	Revertants/plate					
Agent	Dose plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		123	132	116	123.67	8.02
2-AF	20 ug	1356	1428	1398	1394.00	36.17
AP	mg					
	5	196	197	201	198.00	2.65
	2.5	134	121	112	122.33	11.06
	1.25	111	112	110	111.00	1.00
	0.625	131	122	121	124.67	5.51
	0.313	108	113	119	113.33	5.51
Spontaneous	0.00	127	136	131	131.33	4.50
S9-						
DMSO		120	122	130	124.00	5.29
AP	mg					
	5	185	118	118	140.33	38.68
	2.5	114	120	106	113.33	7.02
	1.25	111	110	106	109.00	2.65
	0.625	114	126	100	113.33	13.01
	0.313	123	118	126	122.33	4.04
Spontaneous	0.00	120	122	130	124.00	5.29

Appendix - A3
Ames Test - Strain TA1535
Experiment # 1

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		17	13	10	13.33	3.51
AP	mg					
	5	17	14	15	15.33	1.53
	2.5	14	20	13	15.67	3.79
	1.25	15	16	16	15.67	0.58
	0.625	12	14	16	14.00	2.00
	0.313	15	16	19	16.67	2.08
Spontaneous	0.00	14	8	19	13.66	5.50
S9-						
DMSO		13	13	16	14.00	1.73
Sodium azide	2 ug	956	993	931	960.00	31.19
AP	mg					
	5	15	9	17	13.67	4.16
	2.5	14	10	13	12.33	2.08
	1.25	10	11	16	12.33	3.22
	0.625	19	20	18	19.00	1.00
	0.313	16	19	20	18.33	2.08
Spontaneous	0.00	17	18	14	16.33	2.08

Appendix - A3
Ames Test - Strain TA1535
Experiment # 2

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		23	21	15	19.667	4.163
AP	mg					
	5	19	11	14	14.67	4.04
	2.5	10	10	11	10.33	0.58
	1.25	21	15	17	17.67	3.06
	0.625	11	12	10	11.00	1.00
	0.313	11	12	11	11.33	0.58
Spontaneous	0.00	10	12	8	10.00	2.00
S9-						
DMSO		13	14	15	14.00	1.00
Sodium azide	2 ug	847	977	862	895.33	71.12
AP	mg					
	5	14	15	9	12.67	3.22
	2.5	13	14	18	15.00	2.65
	1.25	16	17	14	15.67	1.53
	0.625	13	15	14	14.00	1.00
	0.313	16	15	13	14.67	1.53
Spontaneous	0.00	10	18	15	14.33	4.04

Appendix - A4
Ames Test - Strain TA1537
Experiment # 1

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		6	7	5	6.00	1.00
AP	mg					
	5	5	8	8	7.00	1.73
	2.5	11	13	9	11.00	2.00
	1.25	13	9	7	9.67	3.06
	0.625	13	9	7	9.67	3.06
	0.313	10	6	8	8.00	2.00
Spontaneous	0.00	7	15	7	7.66	4.01
S9-						
DMSO		9	7	6	7.33	1.53
Aminoacridine	10 ug	134	190	178	167.33	29.48
AP	mg					
	5	9	7	10	8.67	1.53
	2.5	6	10	8	8.00	2.00
	1.25	8	8	9	8.33	0.58
	0.625	11	11	9	10.33	1.16
	0.313	13	9	9	10.33	2.31
Spontaneous	0.00	5	6	18	9.60	7.23

Appendix - A4
Ames Test - Strain TA1537
Experiment # 2

S9+	Revertants/plate					
Agent	Dose/plate	Count 1	Count 2	Count 3	Mean	SD
DMSO		10	9	8	9.00	1.00
AP	mg					
	5	5	7	5	5.67	1.16
	2.5	5	6	9	6.67	2.08
	1.25	5	8	6	6.33	1.53
	0.625	9	8	9	8.67	0.58
	0.313	6	5	7	6.00	1.00
Spontaneous	0.00	6	8	6	6.66	1.15
S9-						
DMSO		8	7	7	7.33	0.58
Aminoacridine	10 ug	98	97	92	95.67	3.22
AP	mg					
	5	8	7	10	8.33	1.53
	2.5	8	5	7	6.67	1.53
	1.25	7	6	7	6.67	0.58
	0.625	7	7	6	6.67	0.58
	0.313	7	5	9	7.00	2.00
Spontaneous	0.00	12	9	3	8.00	4.58

APPENDIX B

RAW DATA OF MOUSE LYMPHOMA MUTAGENESIS ASSAY

Appendix B-1. Raw Data for the Range Finding Experiment with AP

Treatment (µg/mL)	NON-ACTIVATED SYSTEM						S9 ACTIVATED SYSTEM					
	DAY 1			DAY 2			DAY 1			DAY 2		
	Cell counts	Cell conc. 10^6/mL	Daily growth	Cell counts	Cell conc. 10^6/mL	Daily growth	Cell counts	Cell conc. 10^6/mL	Daily growth	Cell counts	Cell conc. 10^6/mL	Daily growth
Medium	185	1.91	6.37	166	1.70	5.67	120	1.22	4.07	150	1.58	5.25
	197			174			124			165		
0.25	167	1.61	5.37	152	1.62	5.38	100	1.06	3.53	191	1.71	5.70
	155			171			112			151		
0.5	175	1.78	5.92	144	1.49	4.97	155	1.61	5.37	155	1.48	4.92
	180			154			167			140		
2.5	114	1.12	3.73	171	1.64	5.47	135	1.40	4.67	193	1.78	5.93
	110			157			145			163		
5	136	1.30	4.33	153	1.47	4.90	40	0.43	1.43	216	1.98	6.58
	124			141			46			179		
25	89	0.85	2.83	133	1.30	4.33	106	1.12	3.72	147	1.40	4.67
	81			127			117			133		
50	100	1.06	3.52	168	1.63	5.43	96	0.98	3.27	139	1.47	4.90
	111			158			100			155		
250	74	0.76	2.53	166	1.54	5.12	124	1.12	3.73	155	1.63	5.43
	78			141			100			171		
500	83	0.85	2.83	145	1.43	4.77	77	0.90	3.00	129	1.34	4.47
	87			141			103			139		
2500	78	0.72	2.38	140	1.43	4.77	40	0.43	1.42	66	0.64	2.13
	65			146			45			62		
5000	75	0.78	2.60	135	1.39	4.63	5	0.04	0.13	20	0.23	0.77
	81			143			3			26		
Pos.	125	1.20	4.00	132	1.37	4.57	95	0.97	3.23	185	1.70	5.67
control	115			142			99			155		

Daily growth = Cell conc./Initial seeding conc. ($3x10^5$ cells/mL)

Appendix B-2. Raw Data for the Mutagenesis Assay Experiment without S9 Activation

	Suspension growth data						Cloning data					
	Day1		Day2		Cumulative	Relative	Number of			Number of		
AP conc. (mg/mL)	Cell Conc. (10^6/mL)	Daily Growth	Cell Conc. (10^6/mL)	Daily Growth	cell counts	suspension growth	viable counts	AVG	Std	Mutant colonies	AVG	Std
Med	1.41	4.70	1.09	3.63	5.12	100.00	169	180.00	14.36	82	89.22	8.00
							174			98		
							196			87		
0.025	1.265	4.22	1.34	4.47	5.65	110.29	190	187.56	2.46	82	81.67	4.33
							187			86		
							186			77		
0.05	1.025	3.42	1.85	6.17	6.32	123.38	125	134.33	13.92	50	56.00	7.54
							128			64		
							150			54		
0.25	1.37	4.57	1.31	4.37	5.98	116.77	175	179.67	8.96	91	72.78	15.75
							174			61		
							190			67		
0.5	1.345	4.48	1.23	4.10	5.51	107.64	181	189.67	21.73	120	103.22	14.74
							173			97		
							214			92		
2.5	0.98	3.27	1.43	4.77	4.67	91.18	128	128.67	1.20	84	81.89	3.37
							130			78		
							128			84		
5	0.81	2.70	1.13	3.77	3.05	59.55	162	164.56	15.66	79	86.67	12.99
							150			79		
							181			102		
DMSO	1	3.33	1.2	4.00	4.00	100.00	164	182.22	24.96	70	70.00	0.47
							211			70		
							172					
EMS	1.19	3.97	1.23	4.10	4.88	121.98	195	197.33	4.67	316	303	13.53
							194			304		
							203			289		

Daily growth = Actual cell conc. / Initial seeding conc (3×10^5)

Cumulative cell count (CCC) = Initial seeding conc (3×10^5) x day 1 growth x day 2 growth

Relative suspension growth = CCC in treated group/ CCC in solvent control

Appendix B-2A. Raw Data for the Mutagenesis Assay Experiment without S9 Activation (Repeat Assay)

	Suspension growth data						Cloning data					
	Day1		Day2		Cumulative	Relative	Number of			Number of		
AP conc. (mg/mL)	Cell Conc. (10^6/mL)	Daily Growth	Cell Conc. (10^6/mL)	Daily Growth	cell counts	suspension growth	viable counts	AVG	Std	Mutant colonies	AVG	Std
Med	2.08	6.93	0.99	3.28	6.38	100.00	151	150.6	3.4	80	72.7	7.5
							147			73		
							154			65		
0.025	2.24	7.47	0.91	3.03	6.79	106.42	179	211.0	31.5	75	85.7	9.3
							242			90		
							212			92		
0.05	1.96	6.53	1.02	3.38	6.63	103.92	221	214.8	5.9	65	68.7	8.1
							213			63		
							210			78		
0.25	2.05	6.83	1.21	4.02	8.23	128.99	196	191.7	5.9	68	74.0	6.6
							185			73		
							194			81		
0.5	2.37	7.9	1.08	3.6	8.53	133.69	141	137.0	4.0	77	74.3	6.4
							133			67		
							137			79		
2.5	2.17	7.24	0.99	3.28	7.13	111.76	197	190.0	10.4	85	85.7	2.1
							195			88		
							178			84		
5	0.95	3.15	1.11	3.68	3.48	54.54	179	173.3	5.1	90	87.0	3.0
							169			84		
							172			87		
DMSO	1.98	6.58	1	3.32	6.55	102.66	171	176.3	6.8	80	72.3	7.1
							174			66		
							184			71		
EMS	1.68	5.6	1.19	3.97	6.66	104.38	100	111.3	14.0	352	388.3	31.6
							107			404		
							127			409		

Daily growth = Actual cell conc. / Initial seeding conc (3×10^5)

Cumulative cell count (CCC) = Initial seeding conc (3×10^5) x day 1 growth x day 2 growth

Relative suspension growth = CCC in treated group/ CCC in solvent control

Appendix B-3. Raw Data for the Mutagenesis Assay Experiment with S9 Activation

	Suspension growth data						Cloning data					
	Day1		Day2		Cumulative	Relative	Number of			Number of		
AP conc. (mg/mL)	Cell Conc. (10^5/mL)	Daily Growth	Cell Conc. (10^5/mL)	Daily Growth	cell counts	suspension growth	viable counts	AVG	Std	Mutant colonies	AVG	Std
Med	1.28	4.27	0.85	2.83	3.63	100.00	181	183.67	6.71	79	94.22	13.24
							191			99		
							179			105		
0.025	1.07	3.57	1.04	3.47	3.71	110.29	168	165.11	2.87	55	56.33	2.33
							166			59		
							162			55		
0.05	0.88	2.93	1.37	4.57	4.02	123.38	252	252.78	0.51	91	104.00	11.15
							253			108		
							253			112		
0.25	0.75	2.50	1.48	4.93	3.70	116.77	251	247.56	14.91	107	108.33	10.27
							231			99		
							261			119		
0.5	0.99	3.30	0.82	2.73	2.71	107.64	196	207.56	20.01	63	67.89	8.76
							231			63		
							196			78		
2.5	0.44	1.47	0.50	1.67	0.73	91.18	309	286.78	22.17	80	82.11	3.08
							265			86		
							287			80		
DMSO	0.91	3.03	0.74	2.47	2.24	59.55	197	193.78	3.02	87	89.00	2.65
							193			92		
							191			88		
3-MCA	0.81	2.70	1.54	5.13	4.16	100.00	186	193.78	10.40	171	176.89	6.17
							206			183		
							189			177		

Daily growth = Actual cell conc. / Initial seeding conc (3×10^5)

Cumulative cell count (CCC) = Initial seeding conc (3×10^5) x day 1 growth x day 2 growth

Relative suspension growth = CCC in treated group/ CCC in solvent control

Appendix B-4. Sizing of TFT Mutants in Nonactivated (-S9) System

AP (mg/mL)	Size (mm) 0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2
Medium	41	28	26	22	20	19	15	9	9	2	0	0
	43	27	25	23	21	21	18	12	10	2	0	0
	41	29	30	26	23	23	16	8	10	3	0	0
0.025	34	26	22	19	15	11	11	10	2	2	0	0
	41	35	29	23	18	14	11	7	2	2	0	0
	32	30	21	17	13	11	10	10	10	4	0	0
0.05	21	17	13	10	9	8	8	3	3	0	0	0
	26	20	14	11	8	7	7	4	4	1	1	1
	25	20	19	15	14	12	9	3	4	1	0	0
0.25	33	26	18	16	14	11	10	8	8	1	0	0
	34	27	21	16	16	14	13	11	10	1	0	0
	46	41	35	27	23	19	16	9	9	2	1	1
0.5	41	31	24	19	16	15	14	11	11	7	2	2
	35	26	20	15	12	11	11	10	10	4	0	0
	34	26	19	14	14	11	9	6	4	4	2	1
2.5	36	26	21	15	13	13	10	4	1	1	0	0
	33	27	22	18	18	16	15	10	10	4	2	2
	42	36	31	30	27	22	18	7	8	1	0	0
5	32	27	19	15	12	12	9	8	8	3	0	0
	35	29	20	14	11	8	7	7	4	2	0	0
	35	29	25	16	11	9	7	5	5	1	0	0
DMSO	41	33	26	20	14	13	13	12	8	2	1	0
	39	30	22	16	12	10	10	5	6	1	0	0
	C*											
EMS	125	107	93	83	73	61	31	5	5	0	0	0
(250nl/mL)	128	114	98	84	71	49	20	4	4	1	1	1
	126	112	98	83	74	50	18	3	0	0	0	0

*Dishes contaminated

Appendix B-5. Sizing of TFT Mutants in S9 Activated System

	Size (mm)											
AP (mg/mL)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2
Medium	41	33	29	27	26	26	22	14	14	3	0	
	45	35	29	25	22	21	16	10	10	5	0	
	39	29	25	22	21	20	16	9	9	3	1	1
0.025	24	17	14	12	11	10	9	9	9	3	0	
	33	28	25	2	21	19	18	13	12	4	2	1
	31	25	20	18	16	15	14	7	5	2	0	
0.05	39	31	22	16	14	15	13	8	1	0		
	57	49	42	32	32	30	26	17	17	4	0	
	48	36	27	21	17	17	15	12	5	4	0	
0.25	42	30	25	21	19	18	16	9	2	3	1	0
	32	26	21	18	16	14	13	8	3	2	0	
	45	36	29	26	24	22	18	11	7	7	1	1
0.5	35	29	24	21	20	19	18	14	13	6	2	1
	36	29	22	16	14	14	12	8	3	3	0	
	37	31	26	24	22	21	21	16	16	11	2	0
2.5	39	33	27	22	20	18	17	14	14	4	1	1
	24	22	19	19	17	16	15	11	11	3	1	1
	29	25	24	21	21	19	18	12	12	7	1	1
DMSO	40	32	27	25	21	18	14	9	9	2	0	
	49	39	32	28	24	22	20	12	12	3	0	
	42	35	28	26	24	23	17	12	12	6	0	
3-MCA	95	84	75	67	58	49	33	17	16	3	0	
(2.5 µg/mL)	99	83	73	62	52	46	32	12	12	1	0	
	99	86	78	71	65	52	37	13	12	1	0	

APPENDIX C

RAW DATA OF *IN VIVO* MOUSE BONE MARROW MICRONUCLEUS TEST

Appendix C. Raw Data of *In Vivo* Mouse Bone Marrow Micronuclei Test (Male Mice)

Treatment (mg/kg)	Code number	Sex	Ratio (PCE/NCE)	Mean ± SD	% MNPCE	Mean ± SD
AP, 1000	06177	M	0.82	0.68 ± 0.04	0.19	0.12 ± 0.06
AP, 1000	06183	M	0.65		0.29	
AP, 1000	06174	M	0.65		0.098	
AP, 1000	06176	M	0.70		0.099	
AP, 1000	06167	M	0.63		0.19	
AP, 500	06175	M	0.45	0.7 ± 0.04	0.196	0.1 ± 0.003
AP, 500	06160	M	0.78		0.29	
AP, 500	06149	M	0.68		0.199	
AP, 500	06171	M	0.76		0.198	
AP, 500	06164	M	0.73		0.19	
AP, 250	06170	M	0.78	0.66 ± 0.05	0.19	0.09 ± 0.003
AP, 250	06150	M	0.43		0.099	
AP, 250	06180	M	0.63		0.099	
AP, 250	06151	M	0.71		0.989	
AP, 250	06185	M	0.6		0.098	
AP, 125	06178	M	0.72	0.59 ± 0.06	0.099	0.13 ± 0.05
AP, 125	06158	M	0.65		0.2	
AP, 125	06163	M	0.69		0.199	
AP, 125	06173	M	0.69		0.199	
AP, 125	06184	M	0.66		0.199	
AP, 62.5	06168	M	0.50	0.62 ± 0.13	0.096	0.17 ± 0.07
AP, 62.5	06156	M	0.62		0.1	
AP, 62.5	06182	M	0.5		0.198	
AP, 62.5	06159	M	0.54		0.099	
AP, 62.5	06168	M	0.52		0.199	

Appendix C. Raw Data of *In Vivo* Mouse Bone Marrow Micronuclei Test (Male Mice)

Treatment (mg/kg)	Code number	Sex	Ratio (PCE/NCE)	Mean ± SD	% MNPCE	Mean ± SD
Control	06157	M	0.39	0.61 ± 0.09	0.198	0.14 ± 0.06
Control	06181	M	0.58		0.198	
Control	06188	M	051		0.29	
Control	06154	M	0.69		0.18	
Control	06161	M	052		0.097	
CP, 20	06155	M	0.64	0.62 ± 0.08	1.6	1.45 ± 0.11
CP, 20	06179	M	0.50		1..48	
CP, 2	06165	M	0.63		1.6	
CP, 20	06187	M	0.56		1.6	
CP, 20	06172	M	0.65		1.67	

Appendix C. Raw Data of *In Vivo* Mouse Bone Marrow Micronuclei Test (Female Mice)

Treatment (mg/kg)	Code number	Sex	Ratio (PCE/NCE)	Mean ± SD	% MNPCE	Mean ± SD
AP, 1000	06894	F	0.7	0.69 ± 0.08	0.091	0.17 ± 0.08
AP, 1000	068118	F	0.75		0.095	
AP, 1000	068130	F	0.66		0.096	
AP, 1000	068106	F	0.67		0.098	
AP, 1000	068116	F	0.63		0.23	
AP, 500	068125	F	0.66	0.68 ± 0.14	0.1	0.16 ± 0.07
AP, 500	068111	F	0.73		0.097	
AP, 500	068123	F	0.68		0.091	
AP, 500	068114	F	0.68		0.1	
AP, 500	068121	F	0.76		0.099	
AP, 250	068104	F	0.74	0.63 ± 0.13	0.098	0.12 ± 0.04
AP, 250	068127	F	0.59		0.093	
AP, 250	068105	F	0.67		0.095	
AP, 250	068115	F	0.67		0.096	
AP, 250	06892	F	0.64		0.089	
AP, 125	06896	F	0.61	0.68 ± 0.03	0.097	0.18 ± 0.05
AP, 125	068120	F	0.63		0.092	
AP, 125	068124	F	0.65		0.18	
AP, 125	068128	F	0.55		0.18	
AP, 125	06895	F	0.51		0.097	
AP, 62.5	068102	F	0.76	0.54 ± 0.05	0.27	0.13 ± 0.05
AP, 62.5	068103	F	0.53		0.2	
AP, 62.5	068119	F	0.72		0.099	
AP, 62.5	068100	F	0.65		0.1	
AP, 62.5	06893	F	0.44		0.19	

Appendix C. Raw Data of *In Vivo* Mouse Bone Marrow Micronuclei Test (Female Mice)

Treatment (mg/kg)	Code number	Sex	Ratio (PCE/NCE)	Mean ± SD	% MNPCE	Mean ± SD
Control	068109	F	0.66	0.54 ± 0.1	0.099	0.19 ± 0.06
Control	06890	F	0.64		0.09	
Control	068126	F	0.53		0.195	
Control	068112	F	0.49		0.195	
Control	06897	F	0.73		0.098	
CP, 20	068110	F	0.55	0.59 ± 0.06	1.6	1.59 ± 0.07
CP, 20	06877	F	0.68		1.4	
CP, 20	068107	F	0.65		1.3	
CP, 20	068117	F	0.57		1.5	
CP, 20	068113	F	0.53		1.5	

APPENDIX D

PROTOCOL FOR THE GENOTOXICITY ASSAYS OF AMMONIUM PERCHLORATE

Protocol for the Genotoxicity Assay of Ammonium Perchlorate

Project No.

Study Title: **Genotoxicity Assay of Ammonium Perchlorate**

Sponsor:
Mike Dourson
TERA, 4303 Hamilton Av.
Cincinnati, OH 45223

Contract Laboratory:
Cellular & Molecular Toxicology Program
ManTech Environmental Technology, Inc.
2 Triangle Dr.
Research Triangle Park, NC, 27709

Proposed Schedule:

1. **Starting Date:** **January 20, 1998**
2. **Completion Date:** **April 24, 1998**
3. **Final Report Date:** **May 26, 1998**

Approvals: Michael J. Dourson — 1·20·98
Study Sponsor

Mark Cunningham — 2/27/98
Project Officer, Perchlorate Study Group — **Date**

[signature] — 1/16/98
Study Director — **Date**
(Cellular & Molecular Toxicology Program)

Protocol for the Genotoxicity Assay of Ammonium Perchlorate

I. Overall Objectives:

Research will be conducted to determine the potential genotoxicity associated with the exposure to ammonium perchlorate (AP), a chemical that is being considered for potential military and space application.

Three short-term genotoxic assays will be used to examine the genotoxicity of ammonium perchlorate, which include:

1. Salmonella/Mammalian microsome reverse mutation assay
   - per EPA (TSCA) Health Efect Testing Guidelines
   (40 CFR 798.5265)

2. Mouse lymphoma assay
   - per EPA (TSCA) Health Efect Testing Guidelines
   (40 CFR 798.5300)

3. *In vivo* mouse bone marrow micronucleus test
   - per EPA (TSCA) Health Efect Testing Guidelines
   (40 CFR 798.5395)

II. Salmonella/Mammalian Microsome Reverse Mutation Assay

II-A. Purpose

The Salmonella/Mammalian microsome reverse mutation system is a microbial assay which measures the reversion from his- (histidine dependent) to his+ (histidine independent) induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

II-B. Background

A reverse mutation assay using Salmonella typhimurium detects mutations in a gene of a histidine requiring strains to produce a histidine independent strain of this organism. A reverse mutation can be achieved by base pair changes, which may occur at the site of the original mutation or at a second site in the chromosome; or by frameshift mutations resulted from the addition or deletion of single or multiple base pairs in the DNA molecule.

In this assay, bacteria are exposed to the test agent with and without a metabolic activation system and plated onto minimum agar medium which is deficient in histidine. After a suitable period of

incubation, revertant colonies are counted and compared with the number of spontaneous revertants in an untreated and / or vehicle control culture. The mutagenicity of the test agents is evident by the increased number of revertants.

II-C. Test Methods

1. Tester strains:

Four tester strains will be used in this assay, which include TA1535 and TA100 for the detection of base pair mutagens, and TA1537 and TA98 for the detection of frameshift mutagens. The tester strains will be obtained from Dr. Bruce N. Ames in University of Califonia, CA.

2. Confirmation of the genotypes of the tester strains:

Following genotypes should be confirmed in each tester strain based on the methods described by Maron and Ames (1983) prior to the mutagenesis study:

a. Requirement of histidine for growth (His-)
b. Sensitivity to Crystal violet (rfa mutation)
c. Sensitivity to U.V. light (uvB mutation)
d. Resistance to ampicillin (R factor)
e. Spontaneous revertant

3. Bacteria growth:

Fresh culture of the tester strains should be used for each assay.
The bacteria are cultured in nutrient broth at 37°C water bath with shaking for 10-12 hours to reach the late exponential or early stationary phase of growth (10^8-10^9 cells per ml).

4. Metabolic activation:

The test compound should be examined both in the presence and absence of an appropriate metabolic activation system. The most commonly used activation system in this assay is S9 mixture, a cofactor supplemented postmitochondrial fraction prepared from the liver of rats treated with enzyme inducers such as Aroclor-1254.

Male Sprague-Dawley rats (b.w. ~200 g) are treated with Aroclor 1254 by i.p. injection at a dose of 200 mg/kg body weight. Five days later animals are sacrificed by cervical dislocation and the livers are collected, homogenized in 0.15 M KCl. The homogenate is centrifuged at 9000 g for 10 minutes. The supernatant is aliquoted and stored at -80°C as the S9 used in the assay.

5. Test agent:

Ammonium Perchlorate will be freshly dissolved in sterile distilled water to the required concentrations. A prescreening test including 5 log doses (with 5 mg/plate as the top dose) will be conducted in TA100 for the dose selection. Toxicity will be evident by a reduction in the spontaneous revertants per plate, and /or a clearing of the background lawn. Five concentrations with adequate intervals will be selected and tested in the mutagenesis.

6. Controls:

In each assay, following concurrent controls will be set up:

a. Negative and solvent controls:

Untreated cultures with and without S9 mixture are set up as negative control. They are used for the measurement of spontaneous revertants, which will serve as the background level of reverse mutation. Appropriate solvent controls will also be included in each assay.

b. Positive control:

Positive controls with known mutagens shall ensure the responsiveness of the tester strains as well as the efficacy of the activation system. Sodium azide (without S9) will be the positive control for TA1535 and TA100. The positive controls for TA98 and TA1537 are 2-aminofluorene (with S9) and 9-aminoacridine (without S9), respectively. The above positive control agents will be dissolved in DMSO.

7. Mutagenesis assay (plate incorporation method):

All dose points (with and without S9 mixture) will be set up in triplicates. 0.1 ml of the culture is added to 2 ml of top agar which is melt and held at 45°C heating block, along with 0.1 ml of the test agent, and 0.5 ml of S9 mixture (in S9+ plates only). The contents aare mixed and then poured onto the surface of a minimum glucose agar plate and spreaded out evenly. The top agar is allowed to solidify and the plates are inverted and incubated at 37°C for 48 hours. The number of revertants per dish is counted by an automatic colony counter.

II-D. Data collection and reporting

The number of revertants per dish will be determined by automatic colony counter, and the results are stored and processed by computer (Excel spreadsheets). Following specific information will be reported for the Salmonella mutagenesis assay: (1) Tester strains used (results of genotypic confirmation), (2) Metabolic activation system used (source, amount, cofactors, method for preparation), (3) Dose levels and the rationale for their selection, (4) Positive and negative controls,

(5) Individual plate counts, means, and standard deviation, and (6) Dose response relationship if applicable.

II-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous revertant frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. A sufficient number of nontoxic concentrations have been tested.

c. The strain-specific positive mutagens significantly increase the revertant in the corresponding strains.

2. Criteria for interpretation:

a. Positive result:

A compound will be considered positive in this assay if a dose-dependent increase in the number of revertants is observed in three concentrations, and the highest increase in TA1535 and TA1537 is equal to three times the spontaneous control value or the highest increase in TA98 and TA100 is equal to two times the spontaneous level (Brusick and Hayes, 1989). Sometimes the precise fold increase will not be necessary if a clear dose-dependent pattern is noted over several concentrations.

A positive result in Salmonella/microsome mutagenesis indicates that under the experimental conditions, the test compound induces point mutation by base changes or frameshift in the genome of this organism.

b. Negative result:

A test agent will be considered negative in this assay if the criteria for positive response are not met, and the tester strains are sensitive to the positive mutagens.

A negative result indicates that under the experimental conditions, the test compound is not mutagenic in Salmonella typhimuriumm

III. Mouse Lymphoma Assay

III-A. Purpose

Mammalian cell culture systems can be used to detect mutations induced by chemical substances. One of the most commonly used mammalian cell mutagenesis system, the L5178Y mouse lymphoma-TK assay detects the mutations at the thymidine kinase locus caused by base pair changes, frameshift and small deletions. Mutant cells deficient in TK due to the forward mutation in the TK locus (from TK+ to TK-) are resistant to the cytotoxic effect of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The mutagenicity of the test agents is indicated by the increase in the number of mutants after treatment.

III-B. Background

Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principle deoxyribonucleotide monophosphates, TMP alone does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition, which serves as a regulator for DNA synthesis. If the TME is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-conpetent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After certain period of expression, the cells are shifted to a selective medium containing the lethal analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). Only the mutant cells (TK-/-) can survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

III-C. Test Methods

1. Cells and culture maintenance:

The L5178Y $TK^{+/-}$ mouse lymphoma cells, clone 3.7.2C are used throughout the study, which were originally obtained from Dr. Donald Clive of former Burroughs Wellcome Co. (Research Triangle park, NC). The cells used in the mutagenesis assay should have a high cloning efficiency and low spontaneous mutation frequency. The cells are maintained as suspension culture in F_{10p} media in culture flasks equilibrated with 5% CO_2.95% air and incubated at 37°C in a rotary shaker.

The cells have a doubling time as 10-11 hours. Each week the cells will be grown in the F_{10p} media containing THMG (thymidine, hypoxanthine, methotrexate and glycine) to select against newly arising TK-/- mutants, and then placed in in the F_{10p} media containing THG (thymidine, hypoxanthine, and glycine) for 1-3 days prior to use in mutagenesis study.

2. Metabolic activation system:

Cells will be exposed to the test agent both in the presence and absence of an appropriate metabolic activation system. Cofactor- supplemented liver S9 from Aroclor-induced rats will be prepared as described by Mitchell et al (1988) and used in each assay.

3. Test agent:

Ammonium Perchlorate will be freshly dissolved with distilled water prior to each use. A preliminary range finding experiment will be conducted using 10 doses over a 3-4 log range with 5000 ug/ml as the top concentration. The procedures for range finding are identical to that used for mutagenesis except that the cultures are terminated after 24-48 hours without further cloning. The toxicity is indicated by the decrease of cell number in the suspension culture comp[ared with that in untreated control. Four to five concentrations will be selected based on the result and used in the mutagenesis assay. The highest dose should produce a low level of survival (approximately 10%), and the survival in the lowest dose should be the same as the negative control.

4. Controls:

Negative control without treatment and positive control with known mutagens should be included in each assay. Ethyl methanesulfonate (EMS, wihtpout S9 mixture) and 3-methylcholanthrene (3-MCA with S9 mixture) will be used as the positive controls. Both mutagens are dissolved in DMSO, and corresponding solvent control will also be included.

5. Mutagenesis assay:

a. Exposure:

Cells ($6x10^6$ cells in 10 ml medium for each culture) are treated with test agents with and without S9 mixture, and incubated at 37°C with rotation for 4 hours. Chemicaals are removed and cells are washed twice by centrifugation then resuspended in non-selective medium at a density of $3x10^5$ cells/ml, and maintained in roller drum for 2 days at 37°C.

b. Expression:

The 2 day maintenance after exposure is the expression period for mutation. During this period, cell density is checked daily and adjusted to $3x10^5$ cells/ml.

c. Cloning:

On the second day of expression, cells are seeded onto soft agar medium to determine the survival and the mutation frequency. For each dose group, 3 cultures containing 200 cells/dish in non-selective medium are set up for viability measurement, another set of 3 cultures with $1x10^6$ cells/dish in selective medium containing TFT are used for mutant counting. Dishes are incubated at 37°C in an atmosphere of 5% CO_2.95% air.

d. Colony counting:

Colonies are counted 11-12 days after cloning using an automatic colony counter. The mutant frequency is calculated and adjusted based on the survival percentage.

III-D. Data collection and reporting

All the original records about cell maintenance, medium and chemical preparation, cell counts, S9 preparation, details for experimental set-up of range finding and mutagenesis assay will be kept in standard forms. Results will be expressed in tabular form which include colony forming efficiecy (CFE %), relative CFE (RCFE), number of mutants, mutation frequency (MF) and relative mutation frequency (RMF) for each culture. Specifically for the mouse lymphoma cell mutagenesis assay , following information will be included in the report: 1. Cells (type, number of cultures, methods for maintenance), 2. test agents (dose selection and rationale). 3. Experimental conditions (incubation temperature, CO_2 concentration, treatment schedule, cell density, metabolic activation system and its preparation, positive and negative controls, length of expression, selective agent and concentration, etc.).

III-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous mutation frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control cultures.

2. Criteria for interpretation:

a. Positive result:

A test agent will be considered to be positive in the mouse lymphoma cell mutagenesis assay if it induces a statistically significant dose-related increase in the mutant frequency, or generates a reproducible and statistically significant increase in the mutant frequency for at least one concentration.

A positive result in mouse lymphoma cell mutagenesis assay indicates that under the experimental conditions, the test compound induces gene mutation in the cells used.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase, or a reproducible and statistically significant increase of the mutant frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce gene mutation in the cells used.

3. Statistical analysis:

The toxicity of the test agent will be indicated by a decrease in CFE (or relative CFE, RCFE), which will be determined as follows:

CFE (%) = number of colonies/number of cells plated x 100%
RCFE = CFE in treated culture/CFE in negative control

The mutagenicity of the test agent will be evident by the increase in mutation frequency (MF, or expressed as relative mutation frequency, RMF) based on the number of mutants and adjusted by the survival fraction of cells:

MF= No. of mutants/ No. of clonable cells x 10^6
RMF = MF in treated culture/MF in negative control

The differences in CFE and MF between control and treated cultures are evaluated by a two-tail Student's t-test. The dose-dependent response is examined by the linear regression.

IV. *In Vivo* Mouse Bone Marrow Micronucleus Test

IV-A. Purpose

The *in vivo* mammalian micronucleus test detects the damage of chromosome or mitotic apparatus caused by chemicals. Polychromatic erythrocytes (PCE) in bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte, the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of the micronucleus is facilitated in the PCEs because they lack the main nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated PCEs in bone marrow of the treated animals.

IV-B. Background

Micronuclei are small particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The clastogenic effect or mitotic apparatus damaging effect of the test agent will be evident by the increased frequency of micronucleated PCEs in the bone marrow.

IV-C. Test Methods

1. Experimental animals:

Swiss CD-1 mice, both sex, 8-10 week old will be used in the study. Animals will be procured from Charles River Laboratory. Five males and five females will be included for each test group. Animals are quarantined for 1 week, and then randomized and assigned to treatment and control groups.

2. Test agent:

Ammonium Perchlorate will be dissolved in distilled water prior to each use and administered by single intraperitoneal (i.p.) injection. In the initial assessment of cytotoxicity, one dose will be used which is the maximal tolerated dose (MTD) or to a maximum of 5000 mg/kg. The cytotoxicity will be judged by a decrease in the ratio of PCEs/NCEs (normochromatic erythrocytes) in the bone marrow. Three doses will be used in the dose-response study.

3. Controls:

Concurrent negative control (without treatment) and positive control (cyclophosphamide, a known micronucleus inducer dissolved in physical saline) will be included. Animals in the negative control group are used for the measurement of background frequency of micronucleated cells, and the

positive control is used to verify the responsiveness of the test system. Saline controls are also included.

4. Dosing and sampling:

Since the *in vivo* pharmacokinetic information of the test agent is unavailable, the experiment will be conducted by the one-dose, one-sampling protocol, one of the most commonly used schedule for this assay. Briefly, the test compounds will be dissolved in distilled water or appropriate solvents, and administered by single intraperitoneal (i.p.) injection. Twenty-four hours after the injection, mice are sacrificed and bone marrow cells are collected.

5. Preparation of bone marrow smears:

The bone marrow cells will be collected and suspended in 3 ml fetal bovine serum. After centrifugation at 1000 rpm for 5 minutes, the pellet will be resuspended with a few drops of FBS, and smears prepared on standard microscopic slides.

6. Staining of the slides:

The slides will be stained the next day of preparation by May-Gruenwald and Giemsa solution as described by Schmid et al (1975). The slides are treated with xylene for 5 minutes and then embedded with coverslips.

7. Micronuclei observation:

The frequency of micronucleated cells are observeded in 1000 polychromatic erythrocytes (PCE) per animal. The PCEs/NCEs ratio is determined by counting 1000 erythrocytes, and used as the indicator of toxicity. Micronuclei are some round bodies in cytoplasm with a diameter of 1/20 to 1/5 of an erythrocyte. They stain intensively, similar to the staining of the main nuclei in the nucleated cells.

IV-D. Data collection and reporting

All the original observation for micronucleated cell frequency and PCEs/NCEs ratio will be recorded in standard scoring forms. Criteria for scoring of micronuclei should be given. Individual data will be presented in a tabular form which includes positive control, negative control, solvent control and treatment groups. The number of PCEs scored, the number of micronucleated PCEs, the percentage of mucronucleated PCEs, and the ratio between PCEs and NCEs will be listed separately.

The test report for micronucleus assay will also include the following specific information: 1. Experimental animals (species, age, body weight, sex, number), 2. Test agent (vehicle, doses and

rationale for dose selection), 3. Treatment and sampling schedule, 4. Toxicity data, 5. Positive and negative controls, 6. Procedures for slide preparation and staining, and 7. Criteria for micronuclei identification.

IV-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The background frequency of micronucleated cells is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control animals.

2. Criteria for interpretation:

a. Positive result:

There are several criteria for determining a positive response in the micronucleus assay. One of which is a statistically significant dose-related increase in the number of micronucleated PCEs. Another criterion is that a reproducible and statistically significant increase in the micronucleated PCE frequency is detected for at least one concentration.

A positive result in micronucleus test indicates that under the experimental conditions, the test compound induces micronuclei by the damages of either chromosome or mitotic apparatus.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase in the number of micronucleated PCEs, or a reproducible and statistically significant increase in the micronucleated PCE frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce micronuclei in the bone marrow of the test species.

3. Statistical analysis:

The differences in the micronucleated PCE frequency and the ratio of PCEs/NCEs among treated and control animals are statistically evaluated by Chi-square analysis, and the dose-dependent

response is examined by linear regression.

V. Identification, Handling and Storage and of the Test Agent

The test agent, ammonium perchlorate will be provided by the sponsor in solid powder form. All the chemical and physical indentifications will be verified and information will be provided by the manufacturer. The compound is highly soluble in water (500 g/liter) and stable below 50°C. It will be kept in sealed dark or opaque glass container and stored at room temperture, avoiding direct sunlight and sudden temperature rise. The primary routes of exposure include skin absorption, ingestion and inhalation. Safety glasses, rubber gloves, and protective clothing is needed for handling.

VI. - Good Laboratory Practice and Quality Assurance

All assays will be conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) Standards as defined in the Federal Register (40 CFR, Part 792, 1992) and the TSCA Test Guidelines (40 CFR 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1992). All the procedures are performed in accordance with the Standard Operating Procedures (SOPs) of ManTech Environmental for the Salmonella/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis and mouse bone marrow cell micronucleus test.

The Quality Assurance Officer of ManTech Environmental Inc. will document inspections on all procedures used in this study. After the initiation of the study, modifications of the protocol will be in the form of Protocol Amendments, which will state the specific modifications and the reasons for the modifications.

VII. Schedule

In the starting phase (about 4 weeks), all the test agents, media, equipments, cells, tester bacteria and animals will be ordered. The genotypes of the tester strains will be confirmed, and cells are maintained. The prescreening studies for dose selection will also be conducted in this period. The 3 genotoxicity assays will be completed within 3 months (12 weeks). Independent confirmatory experiments will be conducted for the mouse lymphoma cell mutagenesis assay and Salmonella/microsome mutagenesis assay depending upon the decision of the sponsor. Another month will be contributed to data analysis and report preparation.

VIII. Reports and Deliverables

An interim technical progress report will be prepared and submitted to the Project Officer, indicating the stage of completion of the requested genotoxic assays on March 10, 1998. The final report will be submitted by May 26, 1998. The study is to be completed by April 24, 1998. The final report constitutes the study's deliverable and ManTech considers acceptance of the deliverable to occur when it is received by TERA.

IX. References:

Brusick D. (1989) In: *Principles and Methods of Toxicology, Second Edition*, A.W. Hayes Ed., Raven Press, NY, p424.

D.M. Maron and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test. Mutation Res., 113, 173-215.

A.D. Mitchell, B.C. Myhr, C.J. Ruddd, W.J. Caspary and V.C. Dunkel (1988) Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Methods used and chemicals evaluated. Environ. Mol. Mutagen., 12 (suppl.13), 1-18.

W.Schmid (1975) The micronucleus test. Mutation Res., 31, 9-15.

Protocol for the Genotoxicity Assay of Ammonium Perchlorate (Revised)

I. Overall Objectives:

Research will be conducted to determine the potential genotoxicity associated with the exposure to ammonium perchlorate (AP), a chemical that is being considered for potential military and space application.

Three short-term genotoxic assays will be used to examine the genotoxicity of ammonium perchlorate, which include:

1. Salmonella/Mammalian microsome reverse mutation assay
   - per EPA (TSCA) Health Efect Testing Guidelines (40 CFR 798.5265)

2. Mouse lymphoma assay
   - per EPA (TSCA) Health Efect Testing Guidelines (40 CFR 798.5300)

3. *In vivo* mouse bone marrow micronucleus test
   - per EPA (TSCA) Health Efect Testing Guidelines (40 CFR 798.5395)

II. Salmonella/Mammalian Microsome Reverse Mutation Assay

II-A. Purpose

The Salmonella/Mammalian microsome reverse mutation system is a microbial assay which measures the reversion from his^- (histidine dependent) to his+ (histidine independent) induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

II-B. Background

A reverse mutation assay using Salmonella typhimurium detects mutations in a gene of a histidine requiring strains to produce a histidine independent strain of this organism. A reverse mutation can be achieved by base pair changes, which may occur at the site of the original mutation or at a second site in the chromosome; or by frameshift mutations resulted from the addition or deletion of single or multiple base pairs in the DNA molecule.

In this assay, bacteria are exposed to the test agent with and without a metabolic activation system and plated onto minimum agar medium which is deficient in histidine. After a suitable period of incubation, revertant colonies are counted and compared with the number of spontaneous revertants in an untreated and / or vehicle control culture. The mutagenicity of the test agents is evident by the increased number of revertants.

II-C. Test Methods

1. Tester strains:

Four tester strains will be used in this assay, which include TA1535 and TA100 for the detection of base pair mutagens, and TA1537 and TA98 for the detection of frameshift mutagens. The tester strains will be obtained from Dr. Bruce N. Ames in University of California, CA.

2. Confirmation of the genotypes of the tester strains:

Following genotypes should be confirmed in each tester strain based on the methods described by Maron and Ames (1983) prior to the mutagenesis study:

a. Requirement of histidine for growth (His^-)
b. Sensitivity to Crystal violet (rfa mutation)
c. Sensitivity to U.V. light (uvrB mutation)
d. Resistance to ampicillin (R factor)
e. Spontaneous revertant

3. Bacteria growth:

Fresh culture of the tester strains should be used for each assay.
The bacteria are cultured in nutrient broth at 37°C in an environmental shaker incubator for 10-15 hours to reach the late exponential or early stationary phase of growth (10^8-10^9 cells per mL).

4. Metabolic activation:

The test compound should be examined both in the presence and absence of an appropriate metabolic activation system. The most commonly used activation system in this assay is S9 mixture, a cofactor supplemented postmitochondrial fraction prepared from the liver of rats treated with enzyme inducers such as Aroclor-1254.

5. Test agent:

Ammonium Perchlorate will be freshly dissolved in sterile distilled water to the required concentrations. A prescreening test including 5 log doses (with 5 mg/plate as the top dose) will be conducted in TA100 for the dose selection. Toxicity will be evident by a reduction in the spontaneous revertants per plate, and /or a clearing of the background lawn. Five concentrations with adequate intervals will be selected and tested in the mutagenesis.

6. Controls:

In each assay, the following concurrent positive and negative controls will be set up:

a. Negative controls (untreated and/or solvent):

Untreated cultures with and without S9 mixture are set up as negative control. They are used for the measurement of spontaneous revertants, which will serve as the background level of reverse mutation.

Appropriate solvent controls will also be included in each assay.

b. Positive control:

Positive controls with known mutagens shall ensure the responsiveness of the tester strains as well as the efficacy of the activation system. Sodium azide (without S9) will be the positive control for TA1535 and 9-aminoacridine (without S9) for TA1537. The positive controls for TA98 and TA100 will be 2-aminofluorene (with S9). The above positive control agents will be dissolved in DMSO.

7. Mutagenesis assay (plate incorporation method):

All dose points (with and without S9 mixture) will be set up in triplicates. 0.1 mL of the culture is added to 2 mL of top agar which is melt and held at 45°C heating block, along with 0.1 mL of the test agent, and 0.5 mL of S9 mixture (in S9+ plates only). The contents are mixed and then poured onto the surface of a minimum glucose agar plate and spread out evenly. The top agar is allowed to solidify and the plates are inverted and incubated at 37°C for 48 hours. The number of revertants per dish is counted manually or by an automatic colony counter.

II-D. Data collection and reporting

The number of revertants per dish will be determined by automatic colony counter, and the results are stored and processed by computer (Excel spreadsheets). Following specific information will be reported for the Salmonella mutagenesis assay: (1) Tester strains used (results of genotypic confirmation), (2) Metabolic activation system used (source, amount, cofactors, method for preparation), (3) Dose levels and the rationale for their selection, (4) Positive and negative controls, (5) Individual plate counts, means, and standard deviation, and (6) Dose response relationship if applicable.

II-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous revertant frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. A sufficient number of nontoxic concentrations have been tested.

c. The strain-specific positive mutagens significantly increase the revertant in the corresponding strains.

2. Criteria for interpretation:

a. Positive result:

A compound will be considered positive in this assay if a dose-dependent increase in the number of revertants is observed in three concentrations, and the highest increase in TA1535 and TA1537 is equal to three times the spontaneous control value or the highest increase in TA98 and TA100 is equal to two times the spontaneous level (Brusick and Hayes, 1989). Sometimes the precise fold increase will not be necessary if a clear dose-dependent pattern is noted over several concentrations.

A positive result in Salmonella/microsome mutagenesis indicates that under the experimental conditions, the test compound induces point mutation by base changes or frameshift in the genome of this organism.

b. Negative result:

A test agent will be considered negative in this assay if the criteria for positive response are not met, and the tester strains are sensitive to the positive mutagens.

A negative result indicates that under the experimental conditions, the test compound is not mutagenic in Salmonella typhimurium.

III. Mouse Lymphoma Assay

III-A. Purpose

Mammalian cell culture systems can be used to detect mutations induced by chemical substances. One of the most commonly used mammalian cell mutagenesis system, the L5178Y mouse lymphoma-TK assay detects the mutations at the thymidine kinase locus caused by base pair changes, frameshift and small deletions. Mutant cells deficient in TK due to the forward mutation in the TK locus (from TK+ to TK-) are resistant to the cytotoxic effect of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The mutagenicity of the test agents is indicated by the increase in the number of mutants after treatment.

III-B. Background

Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principle deoxyribonucleotide monophosphates, TMP alone does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition, which serves as a regulator for DNA synthesis. If the TME is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-conpetent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After certain period of expression, the cells are shifted to a selective medium containing the lethal analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). Only the mutant cells (TK-/-) can survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

III-C. Test Methods

1. Cells and culture maintenance:

The L5178Y $TK^{+/-}$ mouse lymphoma cells, clone 3.7.2C are used throughout the study, which were originally obtained from Dr. Donald Clive of former Burroughs Wellcome Co. (Research Triangle Park, NC). The cells used in the mutagenesis assay should have a high cloning efficiency and low spontaneous mutation frequency. The cells are maintained as suspension culture in F_{10p} media in culture flasks equilibrated with 5% CO_2.95% air and incubated at 37°C in a rotary shaker.

The cells have a doubling time as 10-11 hours. Each week the cells will be grown in the F_{10p} media containing THMG (thymidine, hypoxanthine, methotrexate and glycine) to select against newly arising $TK^{-/-}$ mutants, and then placed in in the F_{10p} media containing THG (thymidine, hypoxanthine, and glycine) for 1-3 days prior to use in mutagenesis study.

2. Metabolic activation system:

Cells will be exposed to the test agent both in the presence and absence of an appropriate metabolic activation system. Cofactor- supplemented liver S9 from Aroclor-induced rats will be used in each assay.

3. Test agent:

Ammonium perchlorate will be freshly dissolved with distilled water prior to each use. A preliminary range finding experiment will be conducted using 10 doses over a 3-4 log range with 5000 ug/mL as the top concentration. The procedures for range finding are identical to that used for mutagenesis except that the cultures are terminated after 24-48 hours without further cloning. The toxicity is indicated by the decrease of cell number in the suspension culture compared with that in untreated control. Four to five concentrations will be selected based on the result and used in the mutagenesis assay. The highest dose should produce a low level of survival (approximately 10–15%), and the survival in the lowest dose should be the same as the negative control.

4. Controls:

Negative control without treatment and positive control with known mutagens should be included in each assay. Ethyl methanesulfonate (EMS, without S9 mixture) and 3-methylcholanthrene (3-MCA with S9 mixture) will be used as the positive controls. Both mutagens are dissolved in DMSO, and corresponding solvent control will also be included.

5. Mutagenesis assay:

a. Exposure:

Cells (6×10^6 cells in 10 mL medium for each culture) are treated with test agents with and without S9 mixture, and incubated at 37°C with rotation for 4 hours. Chemicals are removed and cells are washed twice by centrifugation then resuspended in non-selective medium at a density of 3×10^5 cells/mL, and maintained in roller drum for 2 days at 37°C.

b. Expression:

The 2 day maintenance after exposure is the expression period for mutation. During this period, cell density is checked daily and adjusted to 3 x 10^5 cells/mL.

c. Cloning:

On the second day of expression, cells are seeded onto soft agar medium to determine the survival and the mutation frequency. For each dose group, 3 cultures containing 200 cells/dish in non-selective medium are set up for viability measurement, another set of 3 cultures with 1 x 10^6 cells/dish in selective medium containing TFT are used for mutant counting. Dishes are incubated at 37°C in an atmosphere of 5% CO_2 .95% air.

d. Colony counting:

Colonies are counted 11-12 days after cloning using an automatic colony counter. The mutant frequency is calculated and adjusted based on the survival percentage.

III-D. Data collection and reporting

All the original records about cell maintenance, medium and chemical preparation, cell counts, S9 preparation, details for experimental set-up of range finding and mutagenesis assay will be kept in standard forms. Results will be expressed in tabular form which include colony forming efficiency (CFE %), relative CFE (RCFE), number of mutants, mutation frequency (MF) and relative mutation frequency (RMF) for each culture. Specifically for the mouse lymphoma cell mutagenesis assay , following information will be included in the report: 1. Cells (type, number of cultures, methods for maintenance), 2. test agents (dose selection and rationale). 3. Experimental conditions (incubation temperature, CO_2 concentration, treatment schedule, cell density, metabolic activation system and its preparation, positive and negative controls, length of expression, selective agent and concentration, etc.).

III-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous mutation frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control cultures.

2. Criteria for interpretation:

a. Positive result:

A test agent will be considered to be positive in the mouse lymphoma cell mutagenesis assay if it induces a statistically significant dose-related increase in the mutant frequency, or generates a reproducible and statistically significant increase in the mutant frequency for at least one concentration.

A positive result in mouse lymphoma cell mutagenesis assay indicates that under the experimental conditions, the test compound induces gene mutation in the cells used.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase, or a reproducible and statistically significant increase of the mutant frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce gene mutation in the cells used.

3. Statistical analysis:

The toxicity of the test agent will be indicated by a decrease in CFE (or relative CFE, RCFE), which will be determined as follows:

CFE (%) = number of colonies/number of cells plated x 100%
RCFE = CFE in treated culture/CFE in negative control

The mutagenicity of the test agent will be evident by the increase in mutation frequency (MF, or expressed as relative mutation frequency, RMF) based on the number of mutants and adjusted by the survival fraction of cells:

MF= No. of mutants/ No. of clonable cells x 10^6
RMF = MF in treated culture/MF in negative control

The differences in CFE and MF between control and treated cultures are evaluated by a two-tail Student's t-test. The dose-dependent response is examined by the linear regression.

IV. *In Vivo* Mouse Bone Marrow Micronucleus Test

IV-A. Purpose

The *in vivo* mammalian micronucleus test detects the damage of chromosome or mitotic apparatus caused by chemicals. Polychromatic erythrocytes (PCE) in bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte, the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of the micronucleus is facilitated in the PCEs because they lack the main nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated PCEs in bone marrow of the treated animals.

IV-B. Background

Micronuclei are small particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The clastogenic effect or mitotic apparatus damaging effect of the test agent will be evident by the increased frequency of micronucleated PCEs in the bone marrow.

IV-C. Test Methods

1. Experimental animals:

Swiss CD-1 mice, both sex, 8-10 week old will be used in the study. Animals will be procured from Charles River Laboratory. Five males and five females will be included for each test group. Animals are quarantined for 1 week, and then randomized and assigned to treatment and control groups.

2. Test agent:

Ammonium perchlorate will be dissolved in distilled water prior to each use and administered by gavage for three consecutive days. In the initial assessment of cytotoxicity, at least three doses (up to a maximum of 5000 mg/kg) will be used. The cytotoxicity will be judged by a decrease in the ratio of PCEs/NCEs (normochromatic erythrocytes) in the bone marrow. Five doses will be used in the dose-response study.

3. Controls:

Concurrent negative (saline) control (without treatment) and positive control (cyclophosphamide, a known micronucleus inducer dissolved in physical saline) will be included. Animals in the negative control group are used for the measurement of background frequency of micronucleated cells, and the positive control is used to verify the responsiveness of the test system.

4. Dosing and sampling:

Since the *in vivo* pharmacokinetic information of the test agent is unavailable, the experiment will be conducted by multiple dosing (one dose per day for 3 days), one of the most commonly used schedule for this assay. Briefly, the test compounds will be dissolved in distilled water and administered by gavage dosing. Twenty-four hours after the injection, mice are sacrificed and bone marrow cells are collected.

5. Preparation of bone marrow smears:

The bone marrow cells will be collected and suspended in 2 mL of a mixture of 1% sodium citrate and fetal bovine serum (70:30). After centrifugation at 1000 rpm for 5 minutes, the pellet will be resuspended with a few drops of the mixture, and smears prepared on standard microscopic slides.

6. Staining of the slides:

The slides will be stained the next day of preparation by May-Gruenwald and Giemsa solution as described by Schmid et al (1975). The slides are treated with xylene for 5 minutes and then embedded with coverslips.

7. Micronuclei observation:

The frequency of micronucleated cells are observed in 1000 polychromatic erythrocytes (PCE) per animal by using blind-coded slides. The PCEs/NCEs ratio is determined by counting 1000 erythrocytes, and used as the indicator of toxicity. Micronuclei are some round bodies in cytoplasm with a diameter of 1/20 to 1/5 of an erythrocyte. They stain intensively, similar to the staining of the main nuclei in the nucleated cells.

IV-D. Data collection and reporting

All the original observation for micronucleated cell frequency and PCEs/NCEs ratio will be recorded in standard scoring forms. Criteria for scoring of micronuclei should be given. Individual data will be presented in a tabular form which includes positive control, negative control, solvent control and treatment groups. The number of PCEs scored, the number of micronucleated PCEs, the percentage of micronucleated PCEs, and the ratio between PCEs and NCEs will be listed separately.

The test report for micronucleus assay will also include the following specific information: 1. Experimental animals (species, age, body weight, sex, number), 2. Test agent (vehicle, doses and rationale for dose selection), 3. Treatment and sampling schedule, 4. Toxicity data, 5. Positive and negative controls, 6. Procedures for slide preparation and staining, and 7. Criteria for micronuclei identification.

IV-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The background frequency of micronucleated cells is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control animals.

2. Criteria for interpretation:

a. Positive result:

There are several criteria for determining a positive response in the micronucleus assay. One of which is a statistically significant dose-related increase in the number of micronucleated PCEs. Another criterion is that a reproducible and statistically significant increase in the micronucleated PCE frequency is detected for at least one concentration.

A positive result in micronucleus test indicates that under the experimental conditions, the test compound induces micronuclei by the damages of either chromosome or mitotic apparatus.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase in the number of micronucleated PCEs, or a reproducible and statistically significant increase in the micronucleated PCE frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce micronuclei in the bone marrow of the test species.

3. Statistical analysis:

The differences in the micronucleated PCE frequency and the ratio of PCEs/NCEs among treated and control animals are statistically evaluated by Chi-square analysis, and the dose-dependent response is examined by linear regression.

V. Identification, Handling and Storage and of the Test Agent

The test agent, ammonium perchlorate will be provided by the sponsor in solid powder form. All the chemical and physical identifications will be verified and information will be provided by the manufacturer. The compound is highly soluble in water (500 g/liter) and stable below 50°C. It will be kept in sealed dark or opaque glass container and stored at room temperature, avoiding direct sunlight and sudden temperature rise. The primary routes of exposure include skin absorption, ingestion and inhalation. Safety glasses, rubber gloves, and protective clothing is needed for handling.

VI. Good Laboratory Practice and Quality Assurance

All assays will be conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) Standards as defined in the Federal Register (40 CFR, Part 792, 1992) and the TSCA Test Guidelines (40 CFR 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1995). All the procedures are performed in accordance with the Standard Operating Procedures (SOPs) of ManTech Environmental for the

Salmonella/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis and mouse bone marrow cell micronucleus test.

The Quality Assurance Auditors of ManTech Environmental Inc. will document inspections on all procedures used in this study. After the initiation of the study, modifications of the protocol will be in the form of Protocol Amendments, which will state the specific modifications and the reasons for the modifications.

VII. Schedule

In the starting phase (about 4 weeks), all the test agents, media, equipments, cells, tester bacteria and animals will be ordered. The genotypes of the tester strains will be confirmed, and cells are maintained. The prescreening studies for dose selection will also be conducted in this period. The 3 genotoxicity assays will be completed within 3 months (12 weeks). Independent confirmatory experiments will be conducted for the mouse lymphoma cell mutagenesis assay and Salmonella/microsome mutagenesis assay depending upon the decision of the sponsor. Another month will be contributed to data analysis and report preparation.

VIII. Reports and Deliverables

An interim technical progress report will be prepared and submitted to the Project Officer, indicating the state of completion of the requested genotoxic assays on March 10, 1998. The final report will be submitted by May 26, 1998. The study is to be completed by April 24, 1998. The final report constitutes the study's deliverable and ManTech considers acceptance of the deliverable to occur when it is received by TERA.

IX. References:

Brusick D. (1989) In: *Principles and Methods of Toxicology, Second Edition*, A.W. Hayes Ed., Raven Press, NY, p 424.

D.M. Maron and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test. Mutation Res., 113, 173-215.

A.D. Mitchell, B.C. Myhr, C.J. Ruddd, W.J. Caspary and V.C. Dunkel (1988) Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Methods used and chemicals evaluated. Environ. Mol. Mutagen., 12 (suppl.13), 1-18.

W. Schmid (1975) The micronucleus test. Mutation Res., 31, 9-15.

JUSTIFICATION FOR PROTOCOL REVISIONS

II. Salmonella/Mammalian Microsome Reverse Mutation Assay

1) Section II.C.6.a

The title **Negative and Solvent Controls** is revised to **Negative Controls (untreated and/or solvent)**

2) Section II.C.6.b. Positive control:

The second and third sentences were revised as follows: **Sodium azide (without S9) will be the positive control for TA1535 and 9-aminoacridine (without S9) for TA1537. The positive controls for TA98 and TA100 will be 2-aminofluorene (with S9).**

II. Reasons for Revision

1) This revision is to clarify the language.

2) The original protocol was submitted as a general protocol for costing purposes without detailed review. The protocol was revised at the start of the study to make it consistent with the Standard Operating Procedures actually followed in the laboratory. The revision was to show that 2-aminofluorene with S9 would be used as a positive control for TA100 instead of sodium azide (without S9). Sodium azide was not used as the positive control for TA100 without S9 because it was already used as the positive control for TA1535. Since TA100 is derived from TA1535 and the fact that both detect base-pair mutagens, using sodium azide as the positive control for both TA100 and 1535 would only yield redundant data. In addition, the positive controls that were used adequately showed the sensitivity of the assay, because the positive response with 2-aminofluorene adequately showed that the S9 system was capable of activating mutagens, and also demonstrated the sensitivity of each strain to detect mutagens.The sentence regarding the positive control for TA98 and TA1537 was only for clarifying the ambiguity in the original protocol.

We did not obtain approval for the revised changes in the protocol from the study sponsors because of the time constraints associated with this study.

IV. *In Vivo* Mouse Bone Marrow Micronucleus Test

1. Section IV.C.2. Test agent:

The last part of the first sentence, **single intraperitoneal (ip) injection**, is changed to **gavage for three consecutive days.**

2. Section IV.C.4. Dosing and sampling:

   a. The **one-dose, one-sampling** in the first sentence is changed to **multiple dosing (one dose per day for 3 days)**.

   b. Deleted the following part from the second sentence:
   or appropriate solvents and administered by single intraperitoneal (i.p.) injection
   Revised to:
   and administered by gavage dosing.

3. Section IV.C.7. Micronuclei observation:

   The last part of the first sentence was modified. The frequency of micronucleated cells is observed in 1000 polychromatic erythrocytes (PCES) per animal **by using blind-coded slides.**

Reasons for Revision

1. This revision reflects the change in the dosing route of ammonium perchlorate (see the rationale in #2a).

2. a. Oral gavage is a preferred route of administration (Brusick, 1994), since ammonium perchlorate is soluble in water. Intraperitoneal (i.p.) injection is used only if the test chemical's characteristics preclude its use in gavage dosing (for example, the test chemical is soluble only in DMSO, etc.). Another reason to opt for gavage dosing is based on the assumption that since AP is being evaluated to determine its potential toxicity in humans, one of the possible exposure routes is probably by the oral route. This is indicated in the report of a serious environmental AP contamination in drinking water in the Lake Mead area in Nevada (McKinnon, 1998). Multiple dosing of AP is preferred to make sure that a potential mutagenic effect is not missed from a single dosing, as cell cycle delays can affect the outcome of the experiment.

   b. This revision reflects the change in the dosing route of ammonium perchlorate (see the rationale in #2a).

3. The original protocol omitted to note this procedure. The practice of blind-coding slides is the recommended procedure for eliminating technician bias.

   Because of time constraints for this study, we did not attempt to obtain an approval from the study sponsors for the revised protocol. But these revisions have significantly improved the methods cited in the original protocol.

VIII. **Reports and Deliverables (See attached)**

This section is replaced by the following section:

Technical progress will be communicated informally to the Project Officer prior to completion of genotoxicity assays; because of additional tests run, the completion date is revised to May 22, 1998. The draft final report will be submitted on May 26, 1998. TERA will provide written comments by June 12, 1998, or as soon as possible thereafter. A final report will be submitted by June 26, 1998, or 14 days after receipt of comments from TERA, whichever is later. If extensive changes are needed to address the comments received, an additional price will be negotiated. The final draft report constitutes the study's deliverable, and ManTech considers acceptance of the deliverable to occur when it is received by TERA.

VIII. Reasons for Revision

A revision was made at the request of TERA to include a draft final report. The approved revised schedule for reports and deliverables is included in the signed protocol revision. (See Appendix D.

PROTOCOL REVISION: Page 1 of 2
(Proposed Schedule and Section VIII. Reports and Deliverables are revised.)

Protocol for the Genotoxicity Assay of Ammonium Perchlorate

Project No.

Study Title: Genotoxicity Assay of Ammonium Perchlorate

Sponsor:
Mike Dourson
TERA, 4303 Hamilton Av.
Cincinnati, OH 45223

Contract Laboratory:
Cellular & Molecular Toxicology Program
ManTech Environmental Technology, Inc.
2 Triangle Dr.
Research Triangle Park, NC, 27709

Proposed Schedule (Revised):

1. **Starting Date:** January 20, 1998
2. **Completion Date:** May 22, 1998
3. **Draft Final Report Date:** May 26, 1998
4. **Final Report Date:** June 26, 1998

Approvals:

Michael J. Dourson Date 5.26.98
Study Sponsor (Mike Dourson)

Mike Grant Date 5/29/98
Project Officer (Mike Grant)

Date 5/22/98
Study Director
(Cellular & Molecular Toxicology Program)

VIII. Reports and Deliverables

Technical progress will be communicated informally to the Project Officer prior to completion of genotoxicity assays; due to additional tests run, the completion date is revised to May 22, 1998. The draft final report will be submitted on May 26, 1998. TERA will provide written comments by June 12, 1998, or as soon as possible thereafter. A final report will be submitted by June 26, 1998, or 14 days after receipt of comments from TERA, whichever is later. If extensive changes are needed based on the comments received, an additional price will be negotiated. The draft final report constitutes the study's deliverable and ManTech considers acceptance of the deliverable to occur when it is received by TERA.

All other terms and conditions are unchanged.